The role of the Major Histocompatibility Complex in immune responsiveness in a Holstein Charolais cattle cross population

Rebecca Jayne Baxter

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy
University of Edinburgh
2010

Declaration of Originality

I hereby declare that the research described in this thesis and the thesis itself was composed and originated entirely by myself, except where otherwise stated.

Rebecca Baxter

November 2010

List of Publications

Refereed

Baxter, R., Hastings, N., Law, A., & Glass, E. J. 2008, "A rapid and robust sequence-based genotyping method for BoLA-DRB3 alleles in large numbers of heterozygous cattle", *Animal Genetics*, vol. 39, no. 5, pp. 561-563. (**Chapter 2)**

Baxter, R., Craigmile, S. C., Haley, C., Douglas, A. J., Williams, J. L., & Glass, E. J. 2009, "BoLA-DR peptide binding pockets are fundamental for foot-and-mouth disease virus vaccine design in cattle", *Vaccine*, vol. 28, no. 1, pp. 28-37. (**Chapter 3**)

The Bovine Genome Sequencing and Analysis Consortium (as part of the annotation team), Elsik, C. G., Tellam, R. L., & Worley, K. C. 2009, "The Genome Sequence of Taurine Cattle: A Window to Ruminant Biology and Evolution", *Science*, vol. 324, no. 5926, pp. 522-528.

Book Chapter

Glass, E. J., **Baxter, R**., Leach, R., & Taylor, G. 2010, "Bovine Viral Diseases - The role of host genetics," in *Breeding for Disease Resistance in Farm Animals*, 3rd edn, S. C. Bishop et al., eds., CAB International, pp. 88-140.

Conferences

Baxter, R., Hastings, N., Law, A., & Glass, E. J.2007, "Quantifying the contribution of *BoLA-DRB3* to immune responses in a cattle cross-population" *8th International Veterinary Immunology Symposium 15th-19th August 2007 Ouro Preto, Brazil.*

Baxter, R., Hastings, N., Davies, C.J., & Glass, E. J.2007 "New approaches to genotyping BoLA genes; Sequenced based typing and microarrays for quantifying their role in immune responsiveness" *International Symposium on Animal Genomics for Animal Health 23-25 October 2007 Paris, France*

Baxter, R., Lu, Z., & Glass, E. J. 2008, "Polymorphisms in DRB3 binding pockets affect the immune response to a FMDV peptide in cattle" *XXXI Conference of the International Society for Animal Genetics, 20-24 July 2008 RAI Conference Centre, Amsterdam , The Netherlands.*

Acknowledgements

Firstly, I would like to thank my supervisor Prof. Liz Glass to whom I am greatly indebted for all her support, encouragement and invaluable wealth of knowledge.

I would like to say thank you to my second supervisor Prof. Chris Haley and also to Dave Waddington for all their advice on statistics.

I very much appreciated the opportunity to go to Washington State University to learn the microarray technique designed by Dr. Chris Davies. In addition the further microarrays and support on the analysis when I was back at Roslin was invaluable. I would like to thank both Chris and his technician Dan New.

I am very grateful to those students (Dr. Ronan O'Neill, Dr. Fiona Young and Dr. Despoina Miltiadou) who preceded me and whose hard work meant that this project was possible.

I am very grateful for all the work preformed by Susan Craigmile and the farm staff on the foot-and-mouth disease virus immunisation project.

The bioinformatics advice and skills were provided by Dr. Jan Aerts and the 3D modelling by Dr. Zen Lu.

In addition I would like to acknowledge the staff of Ark Genomics, and in particular David Morrice, for their expert knowledge in sequencing and microarrays.

The project would not have been possible without the funding provided by the Biotechnology and Biological Sciences Research Council and the English Beef and Lamb Executive.

I would like to thank all my friends at Roslin, Susan, Jen, Anna, Richard, Kirsty and Nicola, whose coffee time chats helped to resolve problems as well as providing many entertaining conversations.

Finally, I would like to thank my family for being there to listen, even when they did not understand the science, and for giving me all their encouragement and support. I am especially grateful to my husband Alex who has always believed in me and has been a constant source of love and support. And of course to my beautiful baby girl Niamh who has made me smile after a hard day of writing.

Abstract

Infectious disease is a major issue facing the livestock industry. Further understanding of the role of genetic factors in the observed phenotypic variability of the immune response to pathogens and vaccination could assist in designing improved preventative measures such as more efficacious vaccines and targeted breeding strategies to select for disease resistance. Major candidate genes for controlling immune responsiveness are located within the major histocompatibility complex (MHC). The highly polymorphic classical MHC genes are key determinants in the strength and type of immune response. However, it has proved difficult to establish genotyping approaches to define functionally relevant allelic variations for outbred species such as cattle, not least because the peptide binding clefts (PBC) of classical MHC molecules are highly polymorphic, and the genes within the MHC complex are closely linked.

The overall aim of this project was to investigate the role of MHC genes in immune responsiveness in approximately 200 F_2 and backcross Holstein-Charolais cattle. These animals were generated as part of the Roslin Bovine Genome (RoBoGen) herd, established through a quantitative trait loci (QTL) project, in which a number of phenotypic traits including immune traits were measured. The immune traits included responses to a Foot-and-mouth disease virus (FMDV) peptide, and vaccines against bovine respiratory syncytial virus (BRSV), para-influenza virus 3 (PIV-3) and bovine herpes virus-1 (BHV-1), as well as T cell response to *Staphylococcus aureus*. The immune phenotypes measured included IgG and interferon-γ (IFN-γ) levels and T cell proliferation.

The cattle MHC region, known as bovine leukocyte antigens (BoLA), resides on bovine chromosome 23. The BoLA region contains approximately 200 genes most of which are immune-related. Class II gene polymorphisms were considered to be the most likely to influence the immune traits measured, and the project primarily focused on the best defined gene, *BoLA-DRB3*. A sequence-based typing technique was successfully improved to facilitate genotyping of the PBC of *BoLA-DRB3* in all generations of the RoBoGen herd (approximately 400 animals) and identified 24

distinct alleles. The sequence information obtained also enabled further analysis of the role of defined 'pockets' within the PBC, which directly determine peptide binding affinity.

All datasets were statistically analysed using a residual maximum likelihood (REML) model and it was shown that several of the *DRB3* alleles within the RoBoGen herd had highly significant ($p<0.05$) associations with the immune response to the FMDV peptide. In addition *DRB3* alleles were identified which had significant associations with the response to the respiratory pathogen vaccinations and exposure to *S. aureus*. The pocket analysis also enabled the identification of several amino acid positions within the PBC which were significantly associated with the immune response traits.

In order to explore whether DQ Class II gene polymorphisms also played a role in the variability of responses and whether BoLA Class I-Class II haplotypes could be discerned, microarrays which utilized allele specific oligonucleotides for BoLA Class I and Class II DQ genes were employed. In addition, to investigate whether the number of DQ gene pairs per chromosome influenced the responses, a quantitative polymerase chain reaction (qPCR) assay to determine DQA gene dosage was developed. However, due to the extremely complex nature of the BoLA region both, techniques would require improving to be used for large-scale studies. Nonetheless, information about haplotypes was determined from the microarray results and the qPCR technique lays the foundations for future development to investigate DQ gene dosage.

The MHC region in cattle is very complex due the high level of polymorphisms and gene duplications. It is likely that many genes play a role in the immune response to both pathogens and vaccines. However, from the evidence presented here, polymorphisms in the PBC of *BoLA-DRB3*, particularly within the pockets, are significantly associated with variation in immune response to many different antigens and this information could be exploited in the design of vaccines or breeding cattle for improved disease resistance.

1 General Introduction

1.1 Introduction

The immune system has evolved to protect individuals against infection by pathogenic organisms yet tolerate non-pathogenic organisms and not attack self. Pathogenic organisms, which include bacteria, viruses and parasites, have co-evolved to defeat the hosts' immune system. This conflict between the pathogenic organisms and the individual has resulted in the evolution of an immune system containing many interacting cells and chemicals. The vertebrate immune system is highly complex and involves the interaction of two divisions: the innate and the adaptive immune response.

The innate immune response acts rapidly in a non-specific way against invading pathogens and as such acts as a primary barrier to infection. It also performs a vital role as it initiates and directs the adaptive response. The adaptive immune response on the other hand is slow acting upon first exposure to the pathogen, but because it acts in a specific, targeted manner, subsequent exposure initiates a rapid response.

1.2 Adaptive immune system

Vertebrates have developed highly sophisticated mechanisms to target pathogens in a specific way: the adaptive immune response. After initial exposure to a pathogen the adaptive immune system generates memory cells which are capable of initiating a rapid response upon subsequent exposure to the pathogen. A deeper understanding of the mechanism involved in the adaptive immune response could be utilised for improving resistance to infectious disease.

1.3 Genetics of disease resistance

Increasing disease resistance and improving response to vaccination is a goal of the livestock industry as it would result in improved animal welfare as well as a reduction in costs. An avenue for improvement is the study of the genes and pathways underlying the response to pathogens and vaccines. However, the genetics underlying traits related to host susceptibility or resistance to disease are complex and likely will involve many genes each of which is likely to have a relatively small effect. In humans there have been extensive studies investigating immunogenetics which have found associations with both MHC and non-MHC genes (e.g. cytokine genes and innate immune receptor genes) (reviewed by Hill (1998)).

1.4 Major histocompatibility complex (MHC)

Peter A. Gorer in 1937 is credited with describing the role of histocompatibility in tumour rejection in mice (Gorer 1937). This built on previous congenic mouse strain work by George Snell and his discovery of a histocompatibility locus (Silverstein 2009). However, it was not until the 1970s, when Zinkernagel and Doherty published their work on the restriction of T cells, that the full function of the MHC became apparent (Zinkernagel & Doherty 1974). They discovered that only histocompatible cells can present antigen to T cells as part of the adaptive immune system.

In the mammalian genome the MHC locus can be subdivided into three distinct regions, I, II and III. The Class I and Class II loci encode molecules which are involved in the presentation of antigen to CD8+ or CD4+ T cells respectively (Germain 1994). In addition, in the last 15 years or so, it has become apparent that MHC Class I molecules are also involved in the function of natural killer (NK) cells

(reviewed by Ljunggren & Karre (1990)). In contrast, the Class III region is more diverse, containing immune genes such as tumour necrosis factor α and β, complement proteins as well as other secreted proteins.

All vertebrate genomes have an MHC locus, although the gene composition, order and sequence divergence varies between species (Kelley, Walter, & Trowsdale 2005; Belov et al. 2006). The adaptive immune system is believe to have evolved about 500 million years ago in ancestral jawed vertebrates and one of the most primitive MHC regions is found in nurse sharks (Ohta et al. 2000; Flajnik & Kasahara 2001). Selective pressures such as pathogens and population dynamics have led to considerable diversity involving gene gain and loss in the MHC regions of different animals (Kelley, Walter, & Trowsdale 2005). The human MHC (human leukocyte antigen (HLA)) region spans 3.6 Mb and has 128 functional genes (The MHC sequencing consortium 1999), whilst the chicken has one of the smallest MHC regions with only 19 functional genes in 92 Kb (Kaufman et al. 1999).

The MHC locus is very important in the defence of the host against pathogens and the human MHC has been associated with more diseases than any other region in the genome (Ghodke et al. 2005). Studies have found that genes within the MHC locus are statistically associated with autoimmune diseases, e.g. Diabetes type 1 (Gorodezky et al. 2006), and with susceptibility and resistance to a range of infectious diseases, such as human immunodeficiency virus (HIV) (Kaur & Mehra 2009; Blackwell, Jamieson, & Burgner 2009), however, most of these studies are inconclusive.

1.5 Molecular biology of MHC genes and proteins

1.5.1 Class I

1.5.1.1 Genes

The mouse MHC (H-2) Class I region was the first MHC region to be described, and now complete sequences of the whole MHC locus are available for a variety of species, including both mouse (http://imgt.cines.fr/textes/IMGTrepertoireMHC/Polymorphism/haplotypes/mouse/MHC/Mu_haplotypes.html) and human (The MHC sequencing consortium 1999).

In humans the Class I genes have been well defined. There are several loci: the polymorphic Class Ia (HLA-A, -B,-C) and the less polymorphic or oligomorphic Class Ib (HLA -E, -F and -G). The HLA-B locus is one of the most polymorphic within the human genome with 2069 alleles identified to date (www.ebi.ac.uk/imgt/hla/stats).

In humans the HLA-A, B and C loci have 8 exons: exon 1 encodes the leader peptide, exons 2 and 3 encode the functionally important α1 and α2 domains, exon 4 encodes the α3 domain, exon 5 encodes the transmembrane domain, exons 6 and 7 encode the cytoplasmic tail and exon 8 contributes the last two nucleotides for the C-terminal amino acid and the 3' untranslated region (in HLA-B exon 8 is entirely untranslated) (Crew 1997).

1.5.1.2 Molecules

The classical Class I molecule is a heterodimer consisting of an α chain and a slightly polymorphic β_2 microglobulin chain (not encoded in the MHC locus) (Figure 1.1).

These molecules are found on most nucleated cells and act to distinguish between 'self' and 'non-self'. The α chain is highly polymorphic, with the majority of polymorphisms located within the α1 and α2 domains which form the peptide binding cleft (PBC).

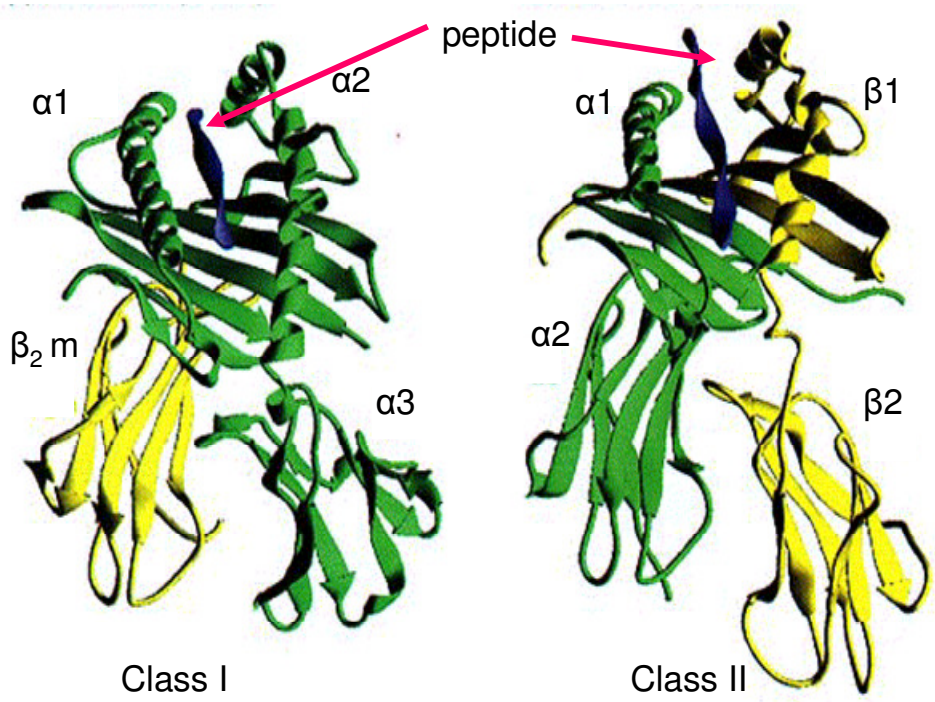


Figure 1.1 MHC Class I and Class II ribbon structure. The green ribbon represents the α chain and the yellow ribbon represents the β chain. The blue ribbon represents a peptide which 'sits' within the binding cleft. (Picture adapted from Stern et al. (1994).

1.5.1.3 Antigen processing and presentation

The main function of the Class I molecules is to present bound peptide to CD8+ T cells. The peptides presented by Class I molecules are obtained from the cytosol of the cell and are derived from either 'self' proteins or intracellular pathogens (viruses, bacteria, or protozoa). As part of the normal machinery of the cell metabolism, proteins that are misfolded or no longer needed are degraded in the cytosol by the

proteasome and translocated by the transporter associated with antigen presentation (TAP) molecules (Momburg & Tan 2002) to the protein synthesis organelle, the endoplasmic reticulum (ER). Intracellular pathogen proteins are also targeted by this pathway as any cell can be invaded by a pathogen. The Class I molecules are assembled within the ER with the assistance of calnexin, calreticulum and ERP57. The MHC Class I molecule in complex with the ER chaperones; calreticulum and ERP57 associate with TAP and tapasin to facilitate peptide binding. The Class I molecule and peptide are transported to the Golgi apparatus and finally to the surface of the cell (reviewed by Cresswell (1994); Pamer & Cresswell 1998).

Under normal circumstances the MHC Class I molecules present 'self' peptides to the T cells which are tolerant to these peptides (Heemels & Ploegh 1995). The Class I molecules also interact with NK cell receptors. Many intracellular pathogens and tumour cells often down regulate MHC Class I molecules to escape the attention of CD8+ T cells. However, an additional layer of defence is provided by NK cells as down regulation of MHC Class I may activate NK cells, potentially resulting in the destruction of the infected/or tumour cell (Trowsdale 2001).

Cross-priming of CD8+ T cells by dendritic cells (DC) allows the presentation of extracellular proteins (e.g. from tumours) or viruses which do not infect APCs to be presented via the Class I pathway, as first described by Bevan (1976).

1.5.1.4 Peptide binding cleft of Class I molecules

The peptide binding cleft (PBC) is formed from 2 α-helices and a β-pleated sheet, which forms a groove for the peptide to 'sit' in (Figure 1.1). The MHC Class I molecule forms a complex with a short cytoplasmically derived (8-11 amino acids in

length) (Engelhard 1994) for presentation to CD8+ T cells. The peptide is anchored by both ends by the pockets that line the peptide binding groove. This results in only part of the peptide being exposed to the T cell receptor (TCR). The PBC pockets are very polymorphic which allows a wide range of peptide to be bound to the molecules.

1.5.2 MHC Class II

1.5.2.1 Genes

The Class II region of the MHC encodes both α and β chains that form the Class II heterodimers. In humans, the classical Class II gene pairs are DRA and DRB, DQA and DQB, DPA and DPB. There is a high degree of polymorphism within the Class II genes. The most polymorphic human Class II gene is *HLA-DRB1* with 809 alleles so far reported (www.ebi.ac.uk/imgt/hla/stats). The human Class II genes have 6 exons: exon 1 encodes the leader peptide, exons 2 and 3 encode the extracellular domains, exon 4 encodes the transmembrane domain, exon 5 encodes the cytoplasmic tail and exon 6 is untranslated.

1.5.2.2 Molecules

The α and β chains are integral membrane glycoproteins which are not ubiquitously expressed, unlike MHC Class I molecules, but are displayed on the surface of antigen presenting cells (APCs), including macrophages, dendritic cells (DC) and B cells (Cresswell 1994). In addition, MHC Class II genes are expressed on other cells in response to IFN-γ (Steimle et al. 1994). The α1 and β1 subunits form the polymorphic PBC (Figure 1.1).

1.5.2.3 Antigen processing

The function of the Class II molecules is to present bound peptide to CD4+ T cells. The peptides which are bound to the Class II heterodimer are primarily derived from internalized proteins. The proteins/pathogens are internalized through the process of phagocytosis by macrophages, DCs and by Fc-mediated uptake by B cells. Once foreign proteins have been internalized they are degraded through the action of enzymes in vesicles called endosomes (Pieters 1997).

An invariant chain (Ii) prevents premature binding of peptides to the newly synthesised Class II molecule in the ER and stabilises the heterodimer. The Class II-Ii chain complex is transported via a vesicle to fuse with the endosome. The enzymes in the endosome cleave the Ii chain leaving a smaller peptide, Class II-associated Ii peptide (CLIP) which sits within the peptide binding cleft. The Class-II like molecule DM binds to the Class II-CLIP complex which catalyses the release of CLIP and allows the binding of the pathogen derived peptide (Weenink & Gautam 1997; Jensen 1998). The Class II-peptide complex then is translocated to the cell surface for presentation (Vogt, Kropshofer & Hammerling 1997) (Figure 1.2).

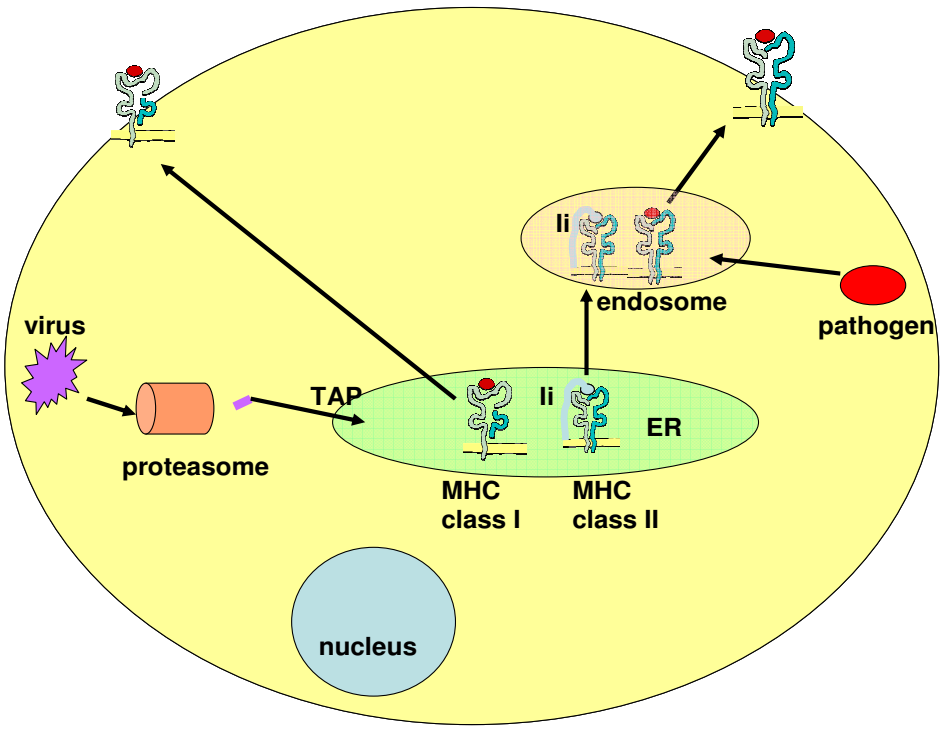


Figure 1.2 Simplified diagram of Class I and II antigen presentation pathways.

Ii: invariant chain, ER: endoplasmic reticulum, transporter associated with antigen processing (TAP): transporter.

1.5.2.4 Peptide binding cleft of Class II molecules

The structure of the Class II molecule is similar to the Class I molecule, with 2 α helices and a β pleated sheet which form the functional PBC. These molecules form a complex with exogenous derived peptides of between 12-19 residues in length, although peptides of up to 30 amino acids have been reported (Engelhard 1994). The PBC has a number of 'pockets' which bind the amino acid side chains of the peptide, these pockets have been numbered 1-9 (Figure 1.3).

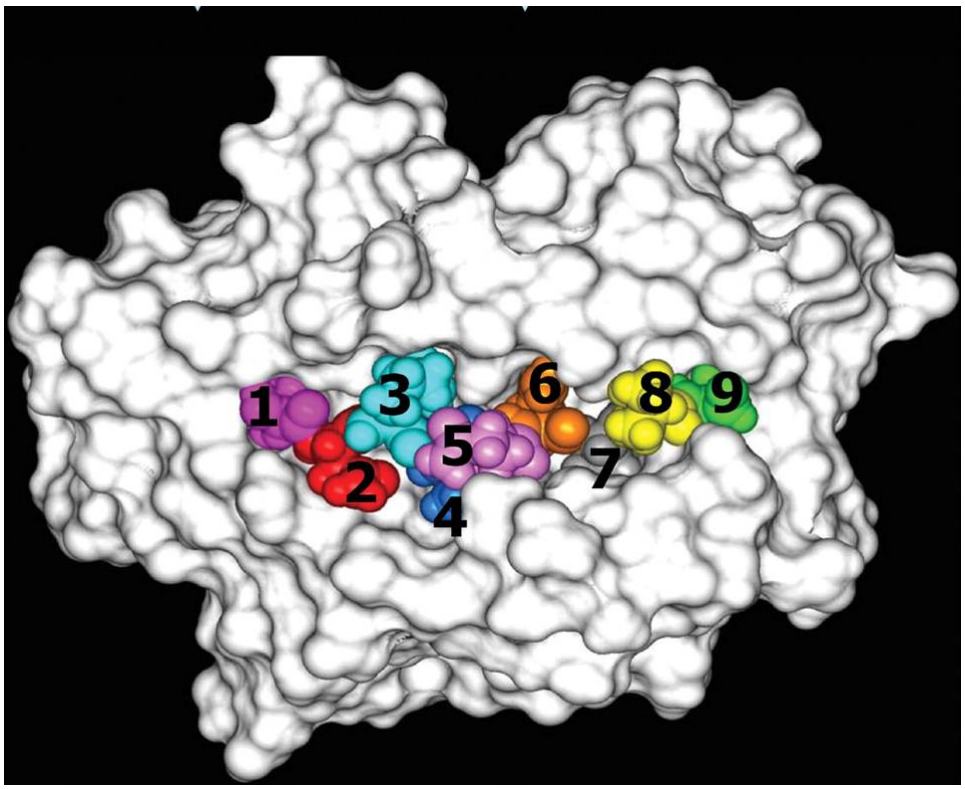


Figure 1.3 HLA-DR molecules peptide binding pockets labelled 1-9 in a predicted molecular surface of peptide binding cleft of HLA-DR4 (Parra-López et al. 2006).

1.5.2.4 MHC Class II antigen presentation

The MHC Class II molecules present peptide on the cell surface to CD4+ T cells. The binding of a peptide to the Class II molecule is dependent on the polymorphisms present in the PBC. However, the binding of a peptide to an MHC molecule does not necessarily mean that an immune response will be generated as the peptide:MHC complex needs to be recognised by T cells through interaction with the TCR.

1.6 T cells

T cells are part of the adaptive immune response and play major roles in both regulation and effector mechanisms of the adaptive immune response which become activated upon exposure to antigen in the form of peptide presented on MHC molecules. T cells are produced by the thymus where they go through a positive and negative selection process to ensure that T cells are tolerant to self-peptides but are reactive to foreign peptides (Kappler et al. 1987).

MHC Class II molecules present to CD4+ T cells which are also known as T helper (Th) cells. T cells are activated through the interaction of TCR-MHC-peptide and co-stimulatory molecules. Once CD4+ T cells are activated they will go through a clonal expansion and assist in the production of humoral immunity through production of cytokines and interaction with B lymphocytes. It was once perceived that activated Th cells could only be sub-divided into two distinct groups depending on their function; either Th1 or Th2 (Mosmann & Coffman, 1989). Th1 cells are involved in the activation of macrophages and production of IFN-γ whilst the Th2 cells are linked with isotype switching of antibodies and production of IL-4. However, other subsets of Th cells have now been described human and mouse, including Th17, Treg, Th9, Th22 and T_{FH} which cross-regulate and have involvement in the immune response (O'Shea & Paul, 2010).

1.6.1 T cell receptor

The activation and expansion of the T lymphocytes is reliant on the recognition of antigen bound to MHC molecules. The TCR is either a αβ or γδ heterodimer, with the αβ TCR interacting with the MHC molecules. The TCR repertoire is highly

diverse which permits the MHC-peptide complexes to interact with a large number of TCRs (Valitutti et al. 1995). In addition, a diverse range of TCRs can recognise a single peptide-MHC complex (Garboczi et al. 1999). The specificity of the TCR is restricted to a small number (three to five) of the peptide amino acid residues which protrude from the peptide-MHC complex (Babbitt et al. 1985). The kinetics of the interaction between the peptide and the TCR would suggest that the affinity of the binding of TCR and peptide-MHC has an impact on the diversity of the clonal expansion of T cells (Baumgartner et al. 2009). The high levels of polymorphism seen within the MHC along with the highly diverse repertoire of the TCR results in an increased possibility that a pathogen will stimulate an adaptive immune response.

1.7 MHC polymorphisms and heterozygote advantage

The genes of the MHC Class I and Class II regions are highly polymorphic and this can be linked to their function. A widely held explanation for the extreme level of diversity observed within the MHC locus is that it is driven by pathogens. A MHC molecule that will bind many peptides at differing affinities would allow the host to maintain its effectiveness against fast evolving pathogens. Therefore a selective advantage would exist for those individuals that carry the widest possible variety of MHC alleles, and the majority of individuals are heterozygous at the MHC loci (Jeffery & Bangham 2000). This is sometimes referred to as “heterozygote advantage”. The main school of thought is that the polymorphism is maintained through balancing selection as a consequence of host–pathogen interactions (Jeffery & Bangham 2000; Woelfing et al. 2009). However there are other theories, including rare allele advantage, whereby an allele which is at a low frequency within a population may confer an advantage if a new pathogen is introduced. If the new

pathogen is particularly virulent then it can exert a strong selective pressure and the rare allele would become more frequent (Jeffery & Bangham 2000).

The polymorphisms observed in the MHC molecules are primarily located in the PBC. The PBC determines whether a peptide binds and will have an impact on the resultant T cell response. The diversity seen in the PBC is thought to be maintained by several mechanisms, including mating preferences (Woelfing et al. 2009). There is no conclusive theory of how diversity is maintained within the MHC region, but it is likely that combinations of these mechanisms are at play in most species.

1.8 Cattle and disease resistance

Cattle are very economically important and they have been domesticated and used by humans for centuries for milk and beef production. Therefore the management and prevention of infectious disease is essential. To a certain extent infectious disease can be controlled by clean management practices, however these do not prevent all outbreaks of disease. The growing numbers of antibiotic resistant pathogens together with the unpopularity of modern pharmaceuticals are directing research to other methods of disease control. Understanding the genetic mechanisms involved in the immune response would help with the management of the infectious disease by providing avenues for improvement in vaccine design as well as implementing breeding programs for disease resistance.

The use of cattle in the study of disease resistance has been limited due to the costs and logistics involved. Cattle studies have mainly focussed on collecting field data, which is subject to other confounding factors. However, with ever improving technologies it became easier to search in an unbiased way for candidate genes using

whole genome scans, for example by using microsatellite markers in linkage studies such as the quantitative trait loci (QTL) study on trypanotolerance (Hanotte et al. 2003). More recently with the advent of dense single nucleotide polymorphism (SNP) markers, loci associated with resistance can be mapped more confidently, and a study is underway at the Roslin Institute on the genetics of tuberculosis resistance in cattle using 700,000 SNP markers (Allen et al. 2010). These studies have the potential to highlight possible important genes for further investigation. Nonetheless, as in the human and mouse studies, the cattle MHC genes remain likely candidates to affect the outcome of infectious disease.

1.9 Vaccination

Vaccination is a practice of priming the immune system to protect an individual against infectious pathogens. It is considered one of the most effective methods of preventing infection. The majority of vaccines usually consist of either killed or inactivated forms of a pathogen, and will therefore contain immunogenic proteins, which have the ability to stimulate the adaptive immune response. There is however substantial problems with current veterinary vaccines in that they are expensive, require the use of adjuvants, often require multiple inoculations, and provide little or no protection to neonates due to the presence of maternal antibodies (reviewed by Shams (2005)). There are also a number of infectious disease to which there are no vaccines available e.g. Mycobacteria. Another problem within veterinary vaccinology is being able to differentiate between those animals with an infection and those which have been vaccinated (DIVA) (Van Oirschot 1999). In the case of foot-and-mouth disease virus (FMDV) it is important for trade to be able to

differentiate animals which have been vaccinated and those that have been exposed to the virus (further discussed in **Chapter 3**).

An ideal vaccine is one in which all animals are protected with few inoculations at a low cost to the industry. In the livestock industry there are a limited number of commercially available vaccines and so it has become necessary to research alternative vaccination protocols, one such avenue for research is using subunit or synthetic vaccines. There are examples of successful use of synthetic subunit vaccine in animals e.g. canine parvovirus (Langeveld et al. 1994) and mink enteritis virus (Langeveld et al. 1995). The advantages of a subunit or synthetic vaccines would be that they are safer and allow for DIVA in those animals which have been vaccinated, however a considerable problem is the variation in immune response to these vaccines. As a peptide would be required to be presented by MHC molecules to provoke an immune response, it is likely that the polymorphism with in the PBC will in part determine the efficacy of a vaccine.

1.10 Bovine MHC

In *Bos taurus* and *B. indicus* the genomic region which harbours the MHC locus maps to chromosome 23 and is collectively named the bovine leukocyte antigen (BoLA) locus. The cattle MHC region has a few interesting attributes including a variable Class I haplotypes and duplicated DQ genes. A number of reviews have been published which have illustrated the complexity of the bovine MHC with high levels of polymorphism and gene duplication (Ellis & Ballingall 1999; Lewin, Russell, & Glass 1999; Ellis 2004; Takeshima & Aida 2006).

1.11 Mapping of the BoLA region

As with many known MHC regions, the cattle BoLA genes are a group of physically linked gene families. The use of radiation hybrid mapping has helped to elicit information on the gene arrangement of the bovine MHC (Band et al. 1998). The bovine MHC Class II went through a large inversion event resulting in two separated regions, designated Class IIa and Class IIb, which are approximately ~2.5 Mb apart, with the Class IIb region closer to the centromere (Childers et al. 2006). This region contains 20 genes, including BoLA-DYA, DYB, DOA, DOB, DMB, DMA and 2 TAP genes, and is indicative of a single inversion event (Childers et al. 2006).

The entire bovine genome has now been sequenced to a 7.1X coverage (The Bovine Genome Sequencing and Analysis Consortium et al. 2009). The BoLA region was included in a community-led effort to annotate the genes in the new assembly. There are predicted to be 154 coding genes (from the genome annotation project) within the BoLA region (~2.5 Mb) which encompasses all 3 MHC classes (personal communication L. Skow). The animals (Domino and Dominette) which were used for the generation of the bovine genome were considered to be homozygous at all loci. However, in the MHC region this proved not to be the case and led to difficulties in assembly and annotation. For example, in the sequence annotation 3 separate loci were identified for *DQA*, but previous research on a number of different breeds has suggested that there is a maximum of 2 loci for *DQA* per haplotype (Andersson & Rask 1988; Russell et al. 1997b). The extra locus is most likely due to the animals being heterozygous for one of the *DQA* genes and the difficulties in an automated system has in arranging sequences of high similarity in complex regions such as the BoLA region.

However, using the bovine genome sequence information (The Bovine Genome Sequencing and Analysis Consortium, et al. 2009) together with the Basic Local Alignment Search Tool (BLAST), it proved possible to 'build' a more detailed map of the BoLA region (Figure 1.4). This figure has been generated using human MHC mRNA sequences (Appendix A.1) listed at http://www.sanger.ac.uk/HGP/Chr6/current_MHC_gene_list.shtml and identifying bovine equivalents (http://www.ncbi.nlm.nih.gov/gene) (with assistance from Dr. Jan Aerts (formerly The Roslin Institute). Those human mRNA sequences which had a bovine equivalent located on BTA23 were taken forward to generate the *in silico* map (n=146) (Appendix A.1) (Figure 1.4). The map is annotated with the human mRNA sequences which were originally used to create the figure.

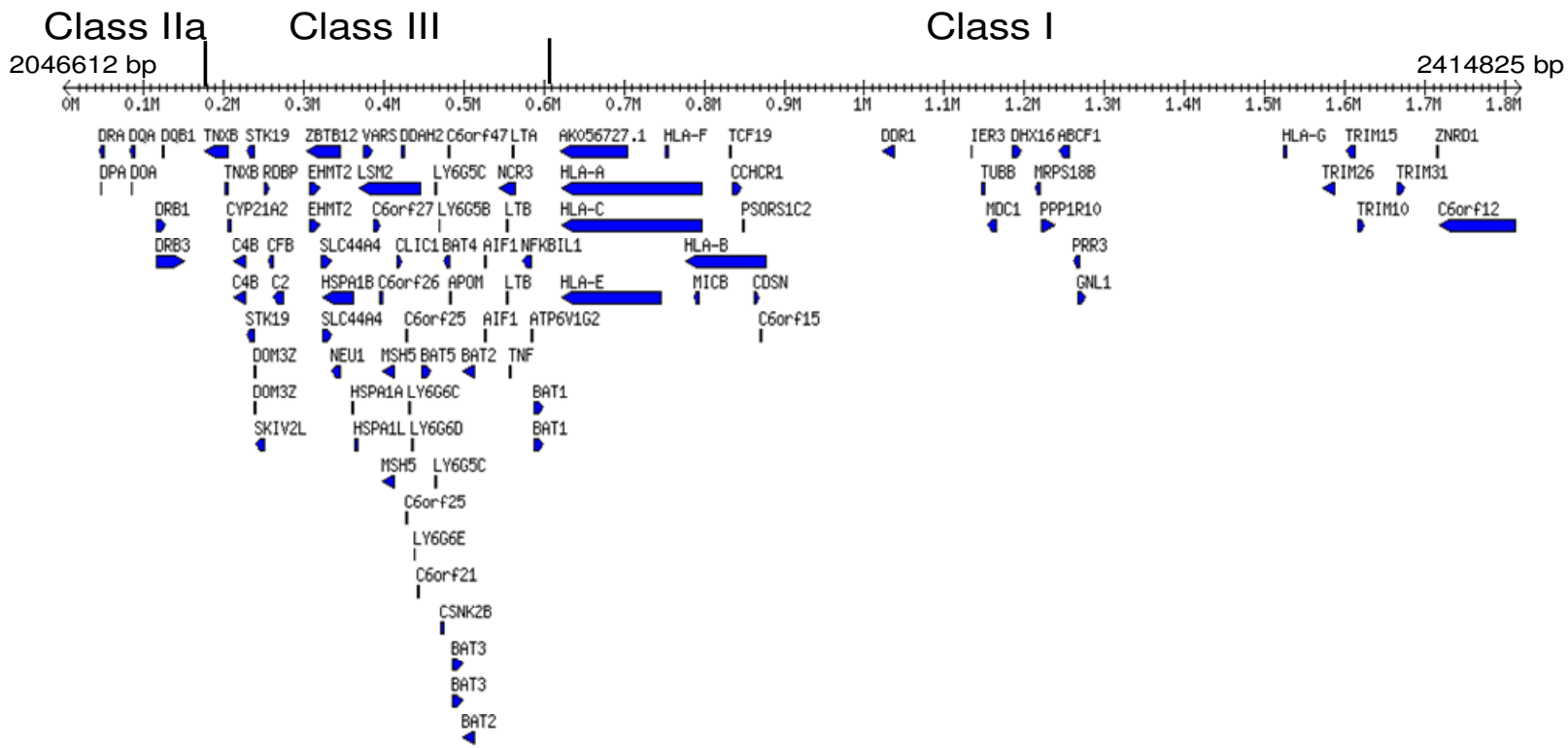


Figure 1.4 *In silico* Map of BoLA Class IIa, III and I regions. The map was generated and annotated using BLAST of human mRNA MHC sequences on the bovine genome with an e-value cut off at -50. The arrows indicate the direction of the genes. The Class IIb region is not represented on this map.

The MHC Class IIb region is not included as full sequence was already available (Childers et al. 2006). Originally the map was intended to be used to locate SNPs across the BoLA region in the hope of generating a SNP-based typing method for the whole BoLA region. However, there are limitations to the building of an *in silico* map in that the accuracy is affected by the stringency of the BLAST threshold, which therefore affects the positioning of the genes, especially in a region where there are high levels of duplication. The quality of the genome sequence and the assembly information also has an effect on the ability to accurately assign gene positions. This map broadly shows the orientation of the Class I, II and III genes, and also highlights the gaps (no sequence data) in the genome sequence (denoted by the extra long genes). As it was primarily human genes which were used to generate the map, it is possible to notice differences, as the bovine genome lacks some of the human genes. For example, in human there are Class II DPA and DPB genes. However on the generated map the DPA maps to the DRA locus indicating high homology.

It was possible with some degree of confidence to locate 75 genes on the *in silico* map of the bovine MHC region, which is 79 genes less than the predicted genes (although 20 will map to the Class IIb region). The *in silico* map has 'hits' for the HLA Class I genes which are all located at similar positions. As the exact position of the BoLA Class I genes are unknown it would be unlikely that this positioning is correct.

This map only gives a broad representation of the BoLA region and more detailed analysis of the gene sequences would be better. Nonetheless this map covers 1.8 Mb of the bovine genome (BoLA ~2.5 Mb), with the most gene dense region being the Class III, and the Class I region showing a lower level density. However, there are

still problems with the genome assembly across the BoLA region and caution should be advised due to potential inaccuracies (Brinkmeyer-Langford et al. 2009).

1.12 BoLA Class I

The BoLA Class I genes are highly polymorphic and a number of haplotypes have shown associations with immune responsiveness (Longeri et al. 1993; Aarestrup, Jensen & Ostergard 1995). The organisation of the Class I region of the bovine MHC is more complicated and less well understood than that of the MHC II genes. The total number of Class I loci in the cattle MHC is estimated to be 20, but this can vary between haplotypes (Holmes et al. 2003). However, evidence indicates that there are only six functional classical MHC Class I loci and the rest are probably pseudogenes. In addition, it appears that of these independent loci, a maximum of 3 genes are expressed per haplotype (Ellis 2004). The complexity of the cattle MHC Class I region is likely due to interlocus recombination and this has assisted in increasing the diversity amongst the alleles (Holmes et al. 2003). It is estimated that the number of alleles within the BoLA Class I region is high, but due to the complexity within the region few studies have been undertaken to type all of the alleles (Babiuk et al. 2007).

The original typing of these molecules used serology with alloantisera (generated from multiparous cattle), and a single A-locus for the Class I molecules was designated (Bernoco et al. 1991), but later techniques demonstrated that this was not accurate, as there is in fact more than 1 locus (Joosten et al. 1992; Ellis et al. 2005). The Class I region has six separate loci but it is still unknown which alleles map to which loci (Birch et al. 2006). There are three polymorphic Class I genes which

appear to be expressed only in certain combinations, with gene 2 being expressed on nearly all the known haplotypes (Birch et al. 2006).

As the Class I molecules present intracellular peptides to CD8+ T cells and interact with NK cells, it is highly likely that the polymorphisms in the Class I genes will have an influence on the immune response, especially to viral infections (Childerstone et al. 1999; Guzman et al. 2008). However, the number of association studies has been limited due to the unknown haplotype structure. Nevertheless 62 alleles have been identified (http://www.ebi.ac.uk/ipd/mhc/bola/) and associations have been found between Class I serotypes and disease, e.g. mastitis and *Mycobacterium bovis* (Longeri et al. 1993; Aarestrup, Jensen, & Ostergard 1995), although it is unclear whether this is because of linkage to BoLA Class II genes.

The gene structure of the BoLA Class I region has not been fully described (Ellis et al. 2005), and has not been annotated on the genome sequence (The Bovine Genome Sequencing and Analysis Consortium et al. 2009). However, the location of three of the Class I genes has been described using a BAC contigs (Di Palma et al. 2002).

1.13 BoLA Class II

The classical BoLA Class II molecules are expressed on APCs. There has been considerable research into the Class II genes and their polymorphisms and this indicates that they play a vital role in disease resistance and susceptibility in cattle. In cattle, DR and DQ are the principal MHC Class II glycoproteins which are expressed. Cattle do not have DP genes, unlike the human MHC region. The genes that code for these proteins are *BoLA-DRA, DRB3* and *BoLA-DQA* and *DQB3* (Ellis & Ballingall 1999).

1.13.1 BoLA DR

The DR Class II molecule is a heterodimer consisting of the monomorphic α chain DRA and the highly polymorphic β chain DRB (Figure 1.5). Of the three *DRB* genes present in the cattle genome, it appears that only the *DRB3* gene is functionally expressed.

DRB3 has six exons. Exon 2 encodes for the PBC and is highly polymorphic (Russell et al. 1997a). The sequencing of *DRB3* exon 2 has revealed 106 different alleles to date (http://www.ebi.ac.uk/ipd/mhc/bola/), with many studies illustrating that the polymorphisms present are significantly associated with the immune response (Dietz et al. 1997; Sharif, Mallard & Sargeant 2000; Sharif, Mallard, & Wilkie 2003; Ballingall et al. 2004; Baxter et al. 2009). Whilst as yet there are no studies demonstrating that the polymorphisms present within the PBC directly affect the outcome of disease in cattle, it would be an interesting avenue to follow.

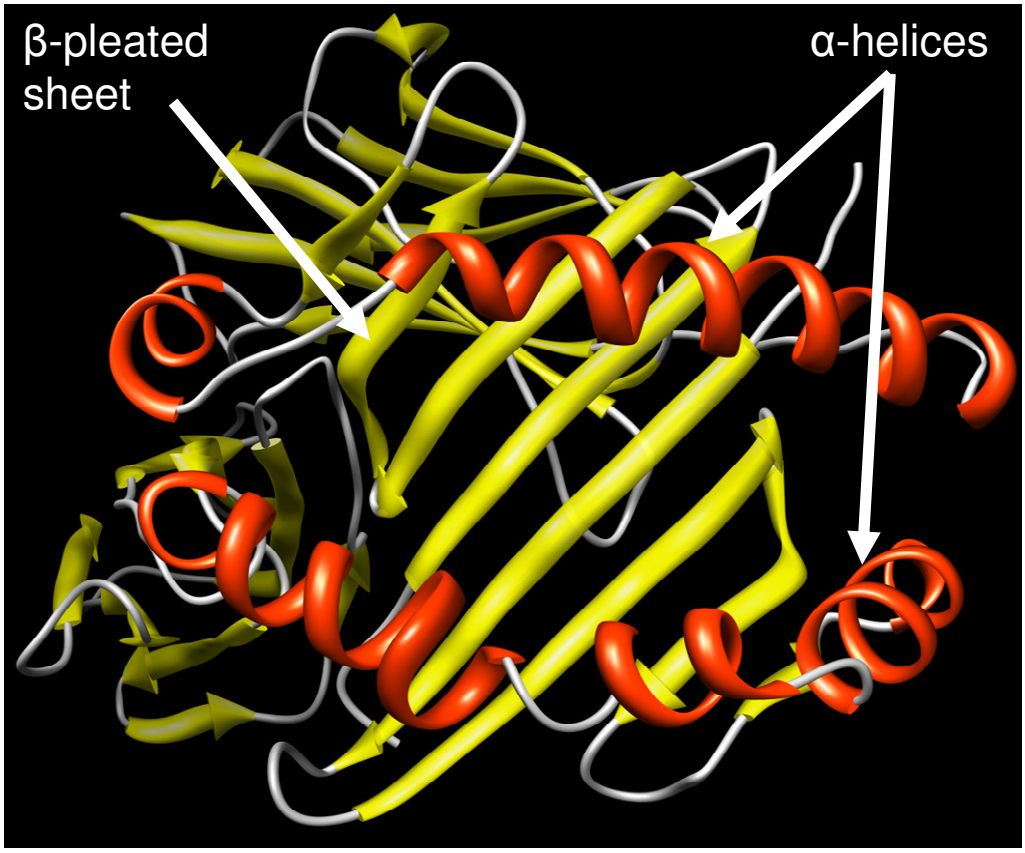


Figure 1.5 3D ribbon structure of a bovine DR molecule. The α- helixes and the β-pleated sheet form the peptide binding cleft. This is where the peptide 'sits' for presentation to T cells. Figure generated from modelling the bovine allele *DRB3*1601* amino acid sequence onto the human *HLA DRB*0101* crystal structure (details of bioinformatic 3D modelling in Chapter 3).

1.13.2 BoLA DQ

The MHC Class II *DQ* genes encode a heterodimer consisting of an α chain: DQA and a β chain: DQB. Unlike the *DR* genes, both are polymorphic. In humans the DQ genes have been associated with autoimmune disease however, no such associations have been noted in cattle. To date 46 *DQA* and 52 *DQB* alleles have been identified (http://www.ebi.ac.uk/ipd/mhc/bola/). This region has a further layer of complexity unique to cattle, as in approximately half of the known haplotypes the *DQ* genes are duplicated (Andersson & Rask 1988; Ballingall, Luyai & Mckeever 1997; Ellis &

Ballingall 1999) and when the genes are duplicated they are expressed (Xu, Park, & Lewin 1994). This has led to difficulties in accurately typing the alleles at the loci. Moreover it has meant that research on polymorphisms in the *DQ* genes has been limited, especially when compared to *DRB3*. Little is known about how *BoLA DQ* polymorphisms in the PBC influence the immune response to pathogens.

From the limited research available, an interesting association is, that animals with *DQB*1804* are susceptible to bovine dermatophilosis (Maillard et al. 2003). However, as this DQ allele was linked to a *DRB3* haplotype (DQB*1804-DRB3.2*09/*45), it was unclear if disease was linked to the effects of DQ or DR polymorphisms. Recently *DQA* heterozygosity has been associated with resistance to mastitis (Takeshima et al. 2008), although again it may be linkage to resistant *DRB3* alleles which accounts for this. However, it is likely that these genes may be important in variation in immune responses to infectious pathogens, as their gene products present peptide to T cells (Glass, Oliver, & Russell 2000; Norimine & Brown 2005).

The DQ molecules may increase the repertoire of peptides that can be presented to T cells through the phenomena of inter- and intra-haplotype pairings, as the α and β chains from either chromosome, and in the case of duplicated haplotypes from either locus, can form functional DQ molecules (Glass, Oliver, & Russell 2000; Norimine & Brown 2005).

1.13.2.1 DQA

It has been hypothesised that the *DQA* genes can be split into 5 different loci, *DQA*1-5, with a maximum of two loci per haplotype (Ballingall, Luyai, & Mckeever 1997;

Gelhaus et al. 1999). The *DQA* loci have not been fully described due to differences between breeds and haplotype structure. It is thought that the DQA haplotype structure consists of either a single *DQA* gene, or DQA1 and DQA2, or DQA2 and DQA3/4, but less is known about the DQA5 locus (Ellis & Ballingall 1999) (Figure 1.6).

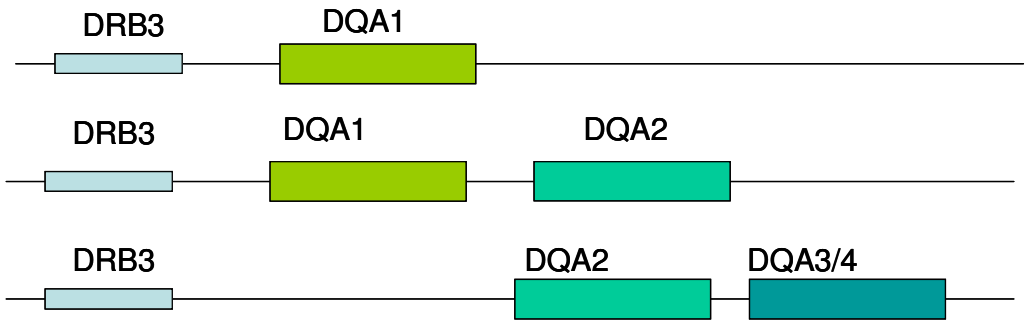


Figure 1.6 *DQA* haplotype structure. Adapted from Ellis & Ballingall (1999).

1.13.2.2 DQB

The *DQB* genes can be subdivided into several loci, *DQB1*-5, although only a maximum of two *DQB* genes are found per haplotype (Nasir et al. 1997; Wang et al. 2005). All *DQB1-5* are thought to be polymorphic (Davies et al. 1997; Takeshima & Aida 2006) and can be duplicated as seen with the *DQA*. Having duplicated *DQA* genes does not necessarily result in duplicated *DQB* genes (Lewin, Russell, & Glass 1999). *DQB3-5* loci have only been reported in *Bos indicus* breeds of cattle.

Little to no research has been reported on the *DQB* alleles or loci and there is only a single study showing a *DQB* allele association with disease (bovine dermatophilosis) (Maillard et al. 2003).

1.14 Binding pockets

Detailed analysis of both human and murine Class II molecules has revealed that the peptide-binding affinity of specific DR alleles, and therefore presentation to T cells, is dependent on the interactions between peptide side chains and 'pockets' within the PBC (Stern et al. 1994; De Groot et al. 2003; Zavala-Ruiz et al. 2004). Several pockets have been identified in the human DR molecule along the PBC (1-9) (Figure 1.3), with pockets 1, 4, 6, 7, 9 and potential pocket 10 each showing high levels of polymorphism (Brown et al. 1993; Zavala-Ruiz et al. 2004). Specific positions and motifs along the PBC in both human (Zerva et al. 1996) and cattle DR (Sharif, Mallard, & Sargeant 2000) molecules have significant association with the immune response to pathogens. The specific polymorphic positions within the PBC have a wide variety of amino acids (Table 1.1). In human HLA-DR molecules, pocket 4 is important for the binding of peptides over and above all the other pockets (Fu et al. 1995).

The DQ molecules have specific binding pockets and again there is high homology between HLA-DQ and BoLA-DQ, although, due to the complexity of the DQ region in cattle, little research has been carried out.

The BoLA-DR molecule has a high degree of homology to HLA-DR and so it is possible to define pockets similar to those found in the human molecules. The identified possible amino acids (using all known *DRB3* allele sequences) within each pocket are detailed in Table 1.1. Studies have indicated that pocket 4 is important in the binding of peptides in the BoLA DR molecule (Zerva et al. 1996; Sharif, Mallard,

& Sargeant 2000). Motifs have been identified within pocket 4 which have either a beneficial or detrimental association with the immune response to different pathogens and vaccine responses. Motifs which have been identified include a lysine deletion at position 65 (Kβ65) (Sitte et al. 2002), which changes the properties of pocket 4, glutamic acid and arginine (E-R) motif at position β70-71 (Xu et al. 1993, Baxter et al. 2009), glutamic acid (E) at position β70 (Sharif, Mallard, & Sargeant 2000) and a glutamic acid, isoleucine, alanine and tyrosine (EIAY) motif at positions β66/67/74/78 (Maillard, Martinez, & Bensaid 1996).

Other binding pockets in human DR molecules (pockets 1, 6, 7, 9 and 10) are also important (Zavala-Ruiz et al. 2004; Cardenas et al. 2005; Bondinas, Moustakas & Papadopoulos 2007) and also likely to play a role in peptide binding in BoLA Class II molecules (Takeshima et al. 2009; Baxter et al. 2009).

Pocket	Position	Amino acid
1	86	C, F, G, M, V
4	13	G,K,R,S
	70	D,E,Q,R
	71	A,E,G,K,R
	74	A,E,N,S,V,Y
	78	V,Y
6	11	A,C,H,L,R,S,T,W,Y
7	28	D,E,H,N,V,Y
	30	C,H,R,S,Y
	47	F,Y
	61	C,L,W
	67	F,I,L,T
9	9	E,K,Q,V
	37	E,F,H,L,N,R,T,Y
	57	A,D,S,V
	60	H,L,Q,Y
10	56	P,Q,R

Table 1.1 Identified polymorphic pockets and amino acid residues at each position in all known *BoLA-DRB3* alleles. A: arginine; C: cysteine; D: aspartic acid; E: glutamic acid; F: phenylalanine, G: glycine; H: histidine; I: isoleucine; K: lysine; L: leucine; M: methionine; N: asparagine P: proline; Q: glutamine; R: arginine; S: serine; T: threonine; V: valine; W: tryptophan; Y: tyrosine.

1.15 BoLA and infectious disease

The BoLA genes have been associated with many diseases (Table 1.2). In addition, quantitative trait loci (QTL) studies have indicated that some genes influencing infectious disease outcome lie on bovine chromosome 23, suggesting that the BoLA region may be involved (Klungland et al. 2001). The alleles of the BoLA-*DRB3* and *DQ* genes have been linked to either resistance or susceptibility to viral and bacterial infections. Modern genotyping methods have allowed the alleles and the binding pockets of *DRB3* to be studied in more detail and associations with response to pathogens have been revealed (Sharif, Mallard, & Sargeant 2000; Baxter et al. 2009).

The BoLA genes clearly have significant associations with disease/vaccine responses, and they are highly polymorphic. A possible avenue for the manipulation of the genetic merits of the BoLA genes could be through the use of selective breeding and has been used successfully for bovine dermatophilosis (Maillard et al. 2003). However, modern cattle breeding in the developed world relies on using a few sires which are considered optimal for production traits. This then limits the diversity seen in the BoLA alleles in the resulting offspring, which possibly may be detrimental on a herd-wide basis. Nonetheless breeding programs can be used to manipulate the BoLA allele frequencies and thus potentially affect the pathogen or vaccine responses within a herd (Ellis & Ballingall 1999).

Disease/ Vaccine Response	**Allele: Resistance; High Response**	**Allele: Susceptible; Low Response**	**Reference**
Bovine Dermatophilosis		*DRB3.2*09*[1], *DRB3.2*45*[1]	Maillard et al. 2003
Bovine Leukaemia Virus	*DRB3.2*16*[1], *DRB3.2*22*[1], *DRB3.2*24*[1], *DRB3.2*27*[1]	*DRB3.2*11*[1], *DRB3.2*23*[1], *DRB3.2*28*[1]	Xu et al. 1993 Lewin, Russell, & Glass 1999
Mastitis (see **chapter 4**)	*DRB3.2*16*[1]	*DRB3*2703*[2]	Sharif, Mallard, & Wilkie 2003 Ledwidge et al. 2001
Bovine leukaemia virus	*DRB*1701*[2]	*DRB3*1501*[2]	Juliarena et al. 2000
Immunization against *Theileria parva*	*DRB3*2703*	*DRB3*1501*	Ballingall et al. 2004
Foot and mouth disease virus peptide vaccine (see **chapter 3**)	*DRB3.2*1*[1], *DRB3.2*3*[1], *DRB3.2*7*[1] *DRB3*1001*[2]	*DRB3.2*12*[1], *DRB3.2*18*[1] *DRB3*24*[1] *DRB3*1601*[2]	Garcia-Briones et al. 2000, Van Lierop et al. 1995, Baxter et al. 2009
tickGARD vaccine	*DRB3*0201*[2], *DRB3*3301*[2]		Sitte et al. 2002

[1] *BoLA DRB3* alleles shown as RFLP nomenclature; [2] the IPD (immuno polymorphism database) nomenclature (http://www.ebi.ac.uk/ipd/mhc/bola/nomen_rules.html)

Table 1.2 *BoLA DRB3* alleles associated with resistance or susceptibility to disease, and with vaccine responses.

1.15.1 BoLA associations with vaccine response

Vaccines harness the adaptive immune system's 'memory' mechanism to protect against a variety of pathogens. Traditionally, vaccines are live-attenuated or inactivated pathogens. However, more recently there has been a move towards new, safer and cheaper synthetic vaccines. Animal to animal variation may be a significant obstacle against the use of these new sub-unit/synthetic vaccines (Sitte et al. 2002). Although potentially effective in the majority of individuals, vaccines may not elicit an effective immune response in all animals (Ellis 2004; Glass 2004). The main difficulty with synthetic vaccines is that they are generally comprised of fewer T-cell epitopes then whole virus pathogens. Therefore it is important that the vaccine-derived peptides are designed to bind to the greatest number of MHC molecules for presentation. Each animal has a different set of MHC alleles and consequently synthetic vaccines need to be designed to allow for this variation. A synthetic vaccine which is not compatible with all BoLA types in the herd could result in a lower response compared to that induced by conventional vaccines (Court et al. 1998). This would leave animals within the herd, which did not respond to vaccination, susceptible to infection.

Currently there is on-going research into improving vaccines, especially in humans (reviewed by Berzofsky, Ahlers & Belyakov (2001)). One possible way to improve a vaccine is to increase the number of possible peptides which can bind to the MHC alleles. Distinct amino acid sequences in the PBC which permit the binding of specific peptides have been identified using a variety of algorithms that predict human T-cell epitopes in vaccine design (Nielsen et al. 2008).

The same methodology could be implemented in the design of livestock vaccines. This would be especially beneficial to combat infections where the current vaccination protocols are far from ideal, e.g. foot and mouth disease (**Chapter 3**). Since the complete genome sequence of many pathogens, including many of importance for livestock such as cattle, are now available, in theory it would be possible to use binding motifs of the BoLA DR molecules to find the most likely important epitopes, thus facilitating design of specific peptide vaccines. The most relevant polymorphisms in the BoLA DR molecules are likely to be those in the binding pockets, although only five of the ten pockets seem to be significant for differences in peptide binding, at least in the limited studies to date (Sharif, Mallard, & Sargeant 2000; Baxter et al. 2009; Yoshida et al. 2009). The advances in *in silico* tools mean that the design of vaccines could be tailored to the PBC and therefore knowing the amino acid sequence of the PBC becomes extremely important (De Groot et al. 2001). Research with pattern-matching programs have shown some success in predicting epitopes for MHC Class I and MHC Class II restricted T cells in cattle (Vordermeier, Whelan, & Hewinson 2003). However, currently there is a lack of information on binding motifs in cattle and so these techniques are possibilities for the future.

1.16 Aims of PhD project

The overall aim of this project was to investigate the role that the MHC genes have on immune responsiveness in a cattle cross Holstein-Charolais population, set up at the Roslin Institute (RoBoGen). The MHC genes are good candidates for influencing the outcome of disease and vaccination due to their integral role in the adaptive

immune response. Prior to the start of this project the RoBoGen animals were immunised with a foot-and-mouth disease virus peptide and two respiratory vaccines, and several immune phenotypes were measured in the herd. These included the antibody response to the two commercial vaccines (Rispoval against bovine respiratory syncytial virus and Imunresp against para- influenza 3 and bovine herpes virus-1), the peripheral blood mononuclear cell (PMBC) proliferative response to *Staphylococcus aureus* and phytohaemagglutinin (PHA), and the IgG1, IgG2, T cell proliferative and IFN-γ responses to a foot-and-mouth disease virus peptide (FMDV15) and Concanavalin A (ConA).

Aims:

- The optimisation and utilisation of typing techniques for the BoLA Class II genes (**Chapters 2** and **5**)
- Identification of the polymorphisms present in *BoLA-DRB3* (**chapter 2**)
- Investigation of the associations between the alleles of the *BoLA-DRB3* gene and measured immune phenotypes (**Chapters 3** and **4**)
- Investigation of the associations between the pocket polymorphisms and measured immune phenotypes (**Chapters 3** and **4**)

1.17 Hypothesis

The genes and polymorphisms present at the *BoLA* loci will have an association with the immune responses measured in the Holstein-Charolais cattle population.

2 Sequence-based typing method for *BoLA-DRB3*

2.1 Introduction

The classical major histocompatibility complex (MHC) Class II genes are highly polymorphic and important in the defence against many pathogens. They have been associated with infectious disease in many host species. Therefore these genes are of interest when studying immune responsiveness. In cattle the BoLA-*DRB3* gene has been linked to a number of immune traits and thus is an ideal candidate for investigating the role of the MHC genes within the context of this project. BoLA-*DRB3* is highly polymorphic and so a sequence-based typing technique was refined and implemented to type the RoBoGen herd.

2.1.1 Background

Human medicine has long been at the forefront of advancements in major histocompatibility complex (MHC) typing techniques. This is mainly due to the requirement for near exact human leukocyte antigen (HLA) type matches for successful organ/bone marrow transplantation. HLA typing procedures have evolved from the early sera-based techniques to the present day DNA-based methods. The advent of the polymerase chain reaction (PCR) technique has permitted allelic differences in HLA genes to be distinguished with a high degree of accuracy, thus enabling the identification of over 3000 alleles present for some of the HLA loci, such as HLA-B and HLA-DRB1 (Erlich, Opelz & Hansen 2001). As well as the practical immediate use for HLA typing there has, in addition, been a sustained focus looking for associations between polymorphisms in the HLA genes and disease outcome, for

example with human immunodeficiency virus (HIV) (Kaur & Mehra 2009) and with leprosy (Cooke & Hill 2001).

Sequence-based typing (SBT) has many advantages over previous techniques, including the ability to investigate single nucleotide polymorphisms (SNPs) as the entire DNA sequence is identified. Having the DNA sequence permits the investigation of the effect of SNPs on the protein structure of the MHC molecules as well as providing the opportunity to discover new alleles.

In the livestock industry there is also a desire to investigate the role of the MHC genes and their polymorphisms in disease outcome, due to the impact that infectious diseases have on both animal welfare and economics (Glass 2004). In order to investigate these associations, it is first necessary to know the variation present within these genes. International BoLA workshops have worked towards providing more information on the variability of the bovine MHC Class I and II genes and molecules (Davies et al. 1994; Davies et al. 1997). Throughout these workshops a number of typing methods were employed: serology (Davies et al. 1994), 1-dimensional isoelectric focusing (Joosten et al. 1992), restriction fragment length polymorphism (RFLP) (Sigurdardottir et al. 1992) and various PCR techniques (Van Eijk, Stewarthaynes & Lewin 1992). A comparison of the different typing methods was reported in the 5th BoLA annual report (Davies et al. 1994). From this international effort it was concluded that it was difficult to use serology and 1-dimensional isoelectric focusing for molecules coded by the Class I region to define the number of alleles, as the antisera recognise more than one gene product.

In addition, the workshops identified many Class II alleles; 63 *DRB3*, 31 *DQA* and 26 *DQB* alleles within the Class IIa region which was mainly achieved though the use of RFLP using human probes. Since then, numerous studies have focused on the typing of a single BoLA Class II gene, *DRB3* (Glass 2004). It has become clear that BoLA *DRB3* is highly polymorphic, with the majority of these polymorphisms located in exon 2 which encodes the peptide PBC of the Class II molecule. To date 106 alleles have been defined for exon 2, using a variety of methods which have gradually improved the resolution of alleles as technology has progressed (http://www.ebi.ac.uk/ipd/mhc/bola). With such a high degree of polymorphism, sequencing of exon 2 is clearly the best way to discriminate between alleles. A historical summary of the various methods for typing is provided below which sets the context for this chapter on sequence-based typing for BoLA *DRB3*. The following work detailed in this chapter has been published (Baxter et al. 2008).

2.1.2 *DRB3* and exon 2

MHC Class II molecules present peptides derived from extracellular sources (e.g. from bacteria) on antigen presenting cells to CD4+ T cells. These peptides are approximately 15-24 amino acids in length and bind to the PBC of the DR or DQ molecule. Exon 2 of the *DRB3* encodes the polymorphisms which affect binding of peptides.

The polymorphisms in the PBC are functionally important as they determine the binding affinity for peptides and thus potentially influence the T cell response to pathogens. In cattle, the DR molecule is a heterodimer formed from a beta chain (DRB3) and a monomorphic alpha chain (DRA). Significant associations have been found between the polymorphisms in the cattle *DRB3* gene and immune

responsiveness to infectious disease, e.g. mastitis and foot-and-mouth disease virus (Maillard, Martinez, & Bensaid 1996; Sharif et al. 1999; Garcia-Briones et al. 2000; Glass 2004). The *DRB3* gene is located within the MHC region on BTA23. The gene contains 6 exons with the second exon coding for the functionally important PBC (Figure 2.1).

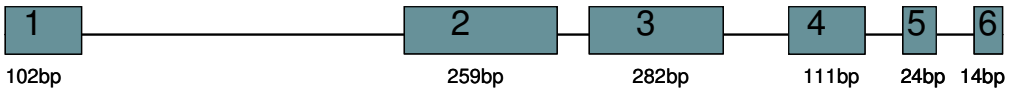


Figure 2.1 Basic *DRB3* gene structure showing 6 exons and the number of base pairs

Due to the high number of polymorphisms within BoLA *DRB3* exon 2, it has been difficult to establish a genotyping approach to define all the functionally relevant allelic variations. Many of the techniques mentioned above were used to report new allelic variants in the various BoLA workshops. However, none of these techniques lend themselves to genotyping large numbers of animals which is generally a pre-requisite for studies investigating associations of gene variants with complex phenotypic traits, such as immune responsiveness or disease resistance. Sequencing, on the other hand, gives reliable and consistent results and enables a thorough examination of all the alleles and more importantly all the polymorphisms within the PBC. This then enables an in-depth examination of how polymorphisms result in conformational changes in the PBC, which would have an affect on peptide binding affinity.

2.1.3 Typing techniques: Class II in cattle

As the PBC coded for by *DRB3* exon 2 is considered to be important in immune responsiveness, there have been many methods used to investigate the polymorphisms, either at the DNA or protein level. More recently, technological advancements have enabled the DNA sequence to be determined.

2.1.3.1 Serology

Serology was one of the original methods of typing MHC both in humans and in cattle. However, in cattle it proved difficult to produce serological reagents through alloimmunisation; as due to the close linkage across the MHC region the allosera produced were multispecific. Despite this, several laboratories matched for Class I and then conducted alloimmunisation. The results were reported at the 4th and 5th BoLA workshops (Bernoco et al 1991; Davies et al. 1994). From this work, five defined Class II specificities were accepted by the bovine research community (Dw designation) and eight others had enough evidence to be designated as clusters (Dc designation) (Davies et al. 1994). However, with the advent of PCR methods, serology has largely become redundant and the classifications have since been superseded with the sequence-based nomenclature (Davies et al. 1997).

2.1.3.2 Isoelectric focusing

A previous method used to identify *DRB3* types was isoelectric focusing. This method showed the Class II polymorphism at the protein level by isoelectric focusing of immunoprecipitated Class II antigens (Joosten et al. 1990; Glass et al. 1992). This technique involves the separation of molecules based on their electric charge on a gel.

However, this method seriously underestimates the polymorphisms present at the gene level, as only 12 variants were accepted by the international community (Davies et al. 1994). Nonetheless the ID-IEF method distinguished *DRB3* alleles that are functionally distinct (Glass, Oliver, & Spooner 1991; Glass et al. 1992).

2.1.3.3 Restriction Fragment Length Polymorphism (RFLP)

PCR-RFLP analysis involves the restriction endonuclease digestion of PCR products. Restriction endonucleases are enzymes which cut DNA at specific nucleotide recognition sites. PCR amplification of a 284 base pair region for all of exon 2, followed by enzyme digestion with *RsaI* (cut-site), *BstYI* (cut-site) and *HaeIII* (cut-site) identified 54 separate *DRB3* alleles (Van Eijk, Stewarthaynes, & Lewin 1992). This technique has also been used to type other BoLA genes, e.g. *DQA* with 13 *DQA1*, 5 *DQA2* and 7 *DQA3* alleles (Ballingall, Luyai, & Mckeever 1997) and *DQB* with 10 *DQB1* and 4 *DQB2* alleles (Sigurdardottir et al. 1992). However, although many *DRB3* alleles were discernable using this technique, it has limitations as the number of detectable alleles is dependent on the presence of suitable enzyme restriction sites and the resolution of fragments by gel electrophoresis.

2.1.3.4 PCR single-strand conformation polymorphism (PCR-SSCP)

PCR-SSCP involves labelled and amplified PCR product which is denatured and resolved by polyacrylamide gel electrophoresis (PAGE). Mutations are detected as altered mobility of separated single strands in the autoradiogram. This method does allow for in depth analysis of MHC genes but has some limitations. For example, the time that it takes to optimise the conditions and the skill required for interpreting the

results reduces efficiency of typing (Hauser 2004). Nevertheless this technique has been used for typing bovine, ovine and equine MHC Class II alleles (Kostia et al. 1998; Pipalia et al. 2004; Diaz et al. 2008).

2.1.3.5 Typing microarray

Another method of typing at the BoLA *DRB3* locus has been reported that involved a microarray-based approach. The typing microarray consisted of 15-22 bp oligonucleotide probes spotted onto a Teflon masked glass slide. Using biotinylated primers, exon 2 was amplified using a PCR reaction, and the product hybridised to the probes (Park et al. 2004). The method identifies *DRB3* alleles, but cannot detect novel sequences and lacks the resolution to clearly distinguish between alleles with high similarity. In **chapter 5** the microarray technique is discussed further.

2.1.3.6 Sequence-based typing

Sequencing of PCR products of exon 2 allows the identification of all known alleles as well as the potential discovery of unknown sequences. The first sequence-based typing (SBT) method for BoLA Class II genes was reported by Takeshima et al. (2001). They used a sequence-specific amplification of eight separate polymorphic groups, which provided sequence information on exon 2 of *DRB3*. This technique was complicated by the requirement for eight separate PCR reactions. A second simpler technique was reported, which involved nested PCRs to amplify the whole of exon 2 which therefore allowed the amplification of all known and unknown alleles (Miltiadou, 2001). One major drawback of this technique was that it involved gel-based sequencing which was time consuming, and only a maximum of 8 samples could be processed per day. Furthermore, the method required the manual identification of bases, which can introduce errors and is subjective. A new method of

sequencing is pyrosequencing, which involves the detection of released pyrophosphate (PPi) during DNA synthesis. This has been used for the detection of equine DRA alleles (Diaz et al. 2008), and has high resolution and so is able to identify many alleles.

2.2 Aim

To develop a fast, reliable and accurate method of typing at the *DRB3* locus in a cross population of Holstein- Friesian and Charolais cattle.

2.3 Materials and methods

2.3.1 Animals

The population was a 2nd generation herd Holstein-Charolais cross. The herd was bred from four pure-bred Charolais sires and eight F1 Charolais-Holstein sires. The 12 sires were used to produce a second generation cross composed of inter-crossed (F1 x F1 n=292), and backcross (Charolais x F1 n=79 and Holstein x F1 n=92) animals, giving a total of 463 animals that were included in this study. The herd was established as an experimental population to investigate quantitative trait loci (QTL) associated with commercially relevant production traits. The animals were born and sampled in 3 separate cohorts from 1999 to 2001 (Figure 2.2).

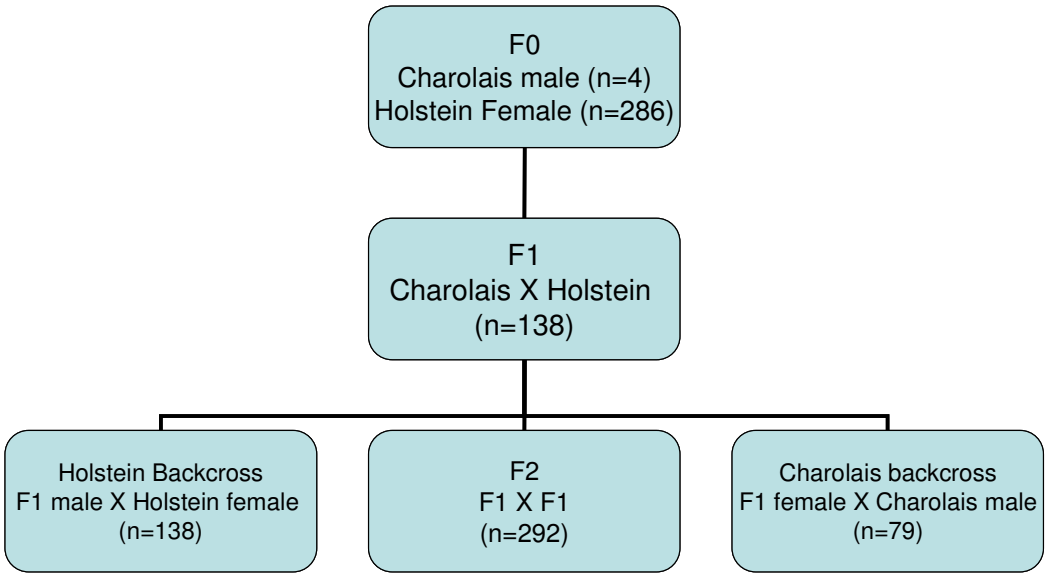


Figure 2.2 RoBoGen breeding and herd structure. F0, F1 and F2 represent the founding animals, the first generation animals and the second generation animals respectively.

2.3.2 DNA

Blood samples were collected from the jugular vein in acid citrate dextrose (ACD). A phenol/chloroform extraction method was used to extract DNA from blood (Miltiadou 2001).

2.3.3 Amplification of *DRB3* exon 2

The amplification of the *DRB3* exon 2 hypervariable region was based on earlier work (Miltiadou 2001). The original method involved using a 2-step nested PCR followed by an internal sequencing reaction. A nested PCR reaction amplified a 319 bp region which was then sequenced using an inner primer set resulting in a sequence of approximately 245 bp. However, I have adapted the method so that only a single set of primers were used, DRB3FRW (5'-CGCTCCGTGAYCAGTCTATCCT-3') and DRB3REW (5'-GTGAGCGCGGGGTGG-3'), which both lie in the intronic region surrounding exon 2 and therefore amplify all of exon 2 (Figure 2.3).

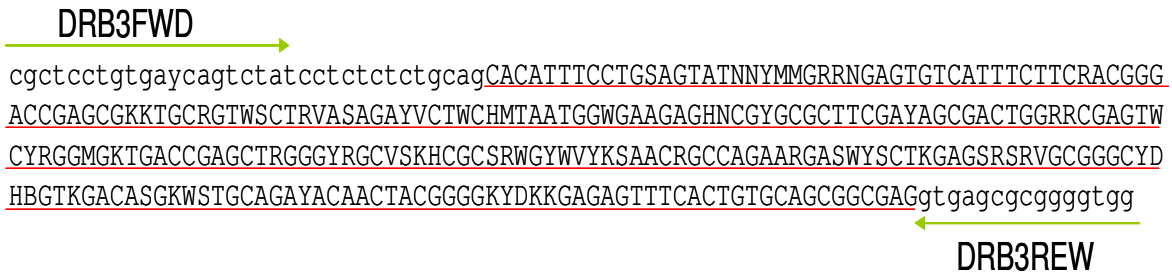


Figure 2.3 The primers DRB3FWD and DRB3REW (marked in green) amplified the hypervariable region (capital letters) by PCR. The red underlined region was used to align the sequence traces.

In detail, approximately 50 ng genomic DNA was used in the PCR reaction in a final volume of 50 µl containing 48 µl PCR mix (50 mM KCl, 10 mM tris-HCl, 1.5 mM $MgCl_2$) (Roche, UK), 200 µM of each dNTP (Bioline, UK) 15pmol each primer and 1 unit of Taq polymerase (Roche UK). The PCR was run for 2.5 min at 94°C, then 30 cycles of 30 sec at 94°C, 30 sec at 57°C and 1 min at 72°C with a final extension of 5 min at 72°C. The PCR product was run on a 1.5% agarose gel and a clear single band of 281 bp was visible (see examples in Figure 2.4). Contaminating salts, unincorporated dNTPs, and primers were removed prior to sequencing using a Montage® PCR µ96 Plate (Millipore, UK).

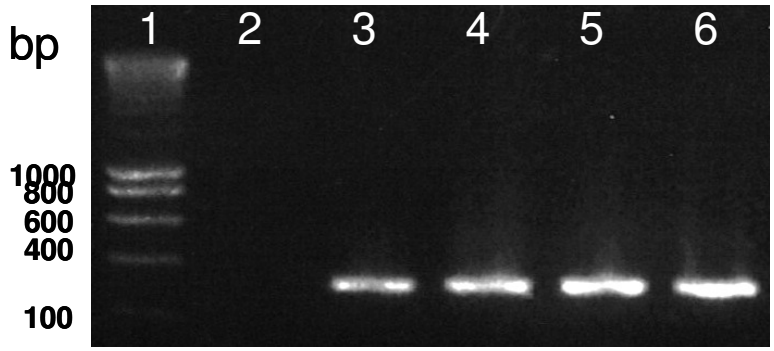


Figure 2.4 Examples of DRB3 exon 2 PCR products from four different animal samples run on a 1.5% agarose gel, showing clear single bands of the correct size of PCR product (281 base pairs).

Lane 1: molecular weight marker hyperladder I (Appendix B.2 for molecular weight); Lane 2: negative control; Lane 3: animal 15095; Lane 4: animal 10775; Lane 5: animal 15057; Lane 6: animal 14810.

2.3.4 Sequencing reaction

All sequencing reactions were performed using a 96-well plate format. Sequencing reactions were achieved using BigDye® v 3.1 terminator sequencing kits (Applied Biosystems, UK) in brief BigDye reaction premix 4X, BigDye sequencing buffer 2X, primers (2.5 pmol), dimethyl sulfoxide (DMSO) 2X, 'cleaned' PCR template and H_2O in a final volume of 10 µl. The DNA was primed with either DRB3FRW or DRB3REV primers to obtain forward and reverse sequence which was analysed on a 3730xl DNA analyser (Applied Biosystems, USA) using the Run 3730 Data Collection v2.0, Sequencing Analysis 5.1.1 (Applied Biosystems, USA) software.

Paired sequence reads were aligned using the BioEdit program (Hall 1999) and visually checked to ensure correct allocation of heterozygous positions using BioEdit. All files were saved in a final FASTA format for use in Haplofinder. Many heterozygous bases were automatically assigned ambiguity codes (Cornish-Bowden 1985), with little requirement for manual intervention.

2.3.5 Haplofinder

Miltiadou et al (2003) used a simple python script (Haplofinder) to assign the *DRB3* alleles. This programme uses all the known alleles to create a list of all possible heterozygous combinations using the IUPAC ambiguity codes (Appendix B.1). The DRB3 sequence (FASTA) files are then inputted into the Haplofinder program which compares them to the list of possibilities. The output from Haplofinder is the two most likely alleles which would give the inputted heterozygous/homozygous sequence. The functionality of the original Haplofinder software reported by Miltiadou et al (2000) has been extended by adding a pre-processing step ('quicklook') which first scans the input sequences and reports differences from the consensus sequence. This removes the requirement for manual examination of input sequences and identifies putative polymorphic bases, which improves the efficiency of processing large numbers of samples (http://www.bioinformatics.roslin.ac.uk/haplofinder/haplofinder.py).

2.4 Results

The animals from the F2 and backcrosses (409) were successfully sequenced at the *DRB3* locus (Appendix B.1). It was not possible to sequence 54 animals due to quality issues with the genomic DNA or lack of DNA sample in this population. Miltiadou et al (2000) also typed 128 of the F2 animals and the 12 founder sires by SBT. The sires had also been typed using PCR-RFLP for confirmation. All the F2 animals were re-typed with the new method to verify the alleles. An example of a heterozygous animal from the RoBoGen herd (Figure 2.5) shows the clarity of the sequence read and the ease with which heterozygous bases are identified. Twenty-two distinct alleles were identified in the population and their frequencies are shown in Figure 2.6.

The allele with the highest frequency was **2707*, which resulted from the herd design which involved four F0 Charolais sires, three of which had this allele. The Charolais backcross population had a frequency of 41% for the **2707* allele (Figure 2.7). In contrast, in the Holstein backcross population the most common alleles were **1101* at 19% frequency, and **2707* at 13% frequency (Figure 2.7). The F2 population had more allelic diversity, but again due to the herd design, **1101* and **2707* (6% and 4% respectively) remained at the highest frequency (Figure 2.7). The Charolais backcross population only had 12 different alleles, although again this is most likely a reflection of the breeding structure from the 4 purebred Charolais sires. The **2707* allele seemed to have originated only from the Charolais sires. The Holstein backcross population had more alleles (18), although some of these were very similar, e.g. **1101* and **1102* only differed by 2 base pairs.

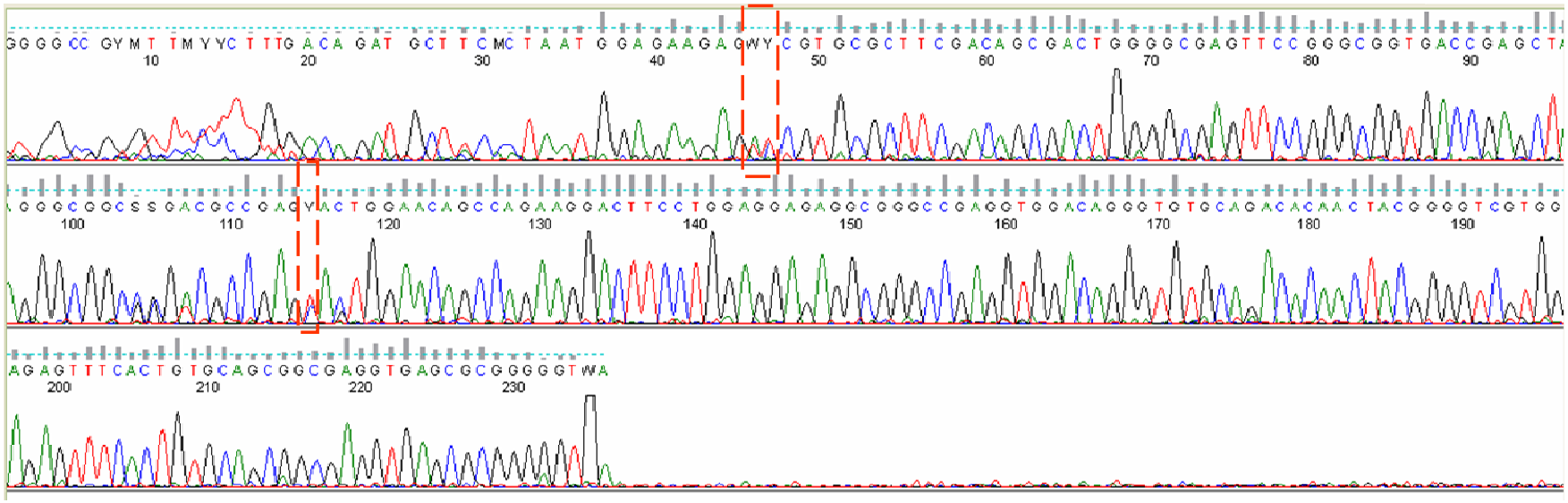


Figure 2.5 Sequence trace of *DRB3* exon 2 hypervariable region from animal 14910 showing forward sequence read, red boxed bases are heterozygous peaks and are designated with heterozygous codes as stated by IUPAC (www.IUPAC.org) nomenclature (Appendix B.1) . The sequence trace is for animal 14910 which was typed as heterozygous: *DRB3*2707* and **0701.*

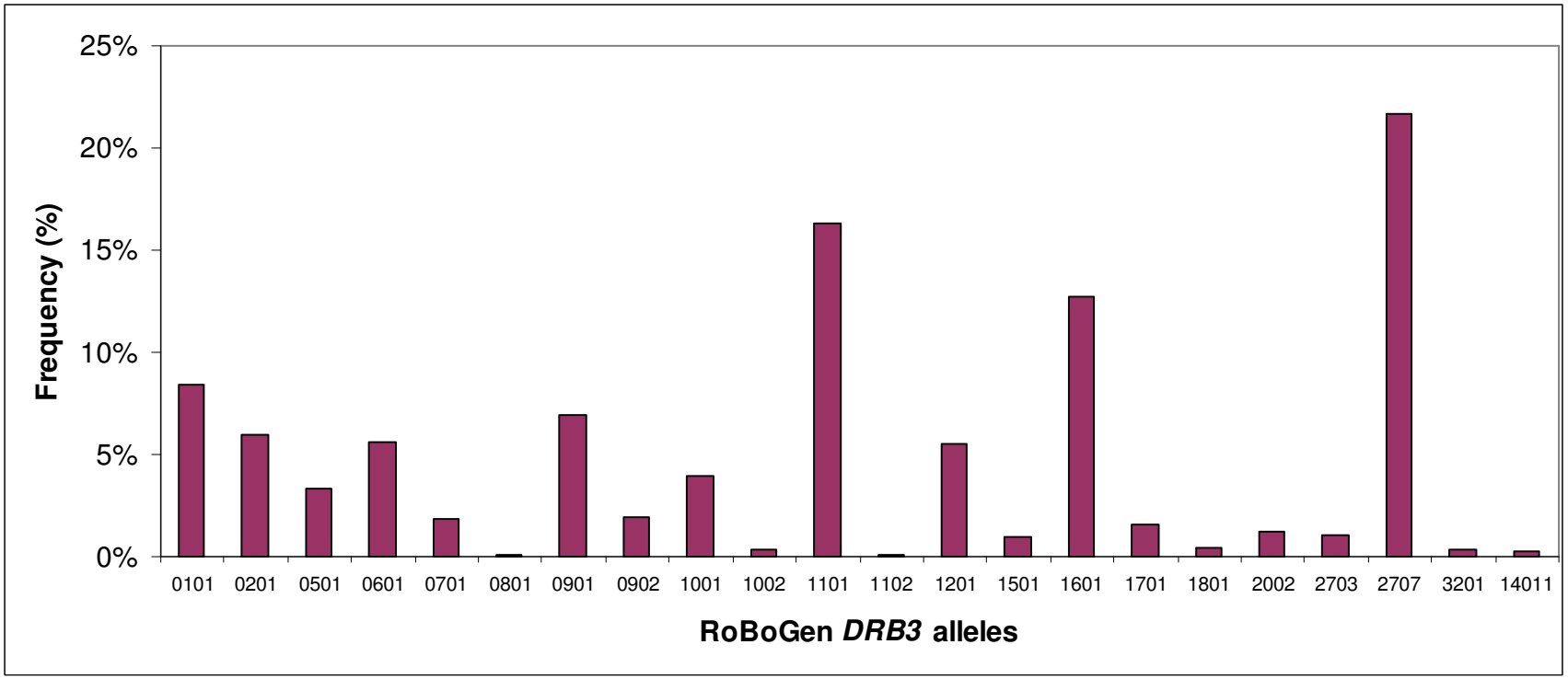


Figure 2.6 Alleles identified and their frequencies within the RoBoGen Charolais-Holstein cross population.

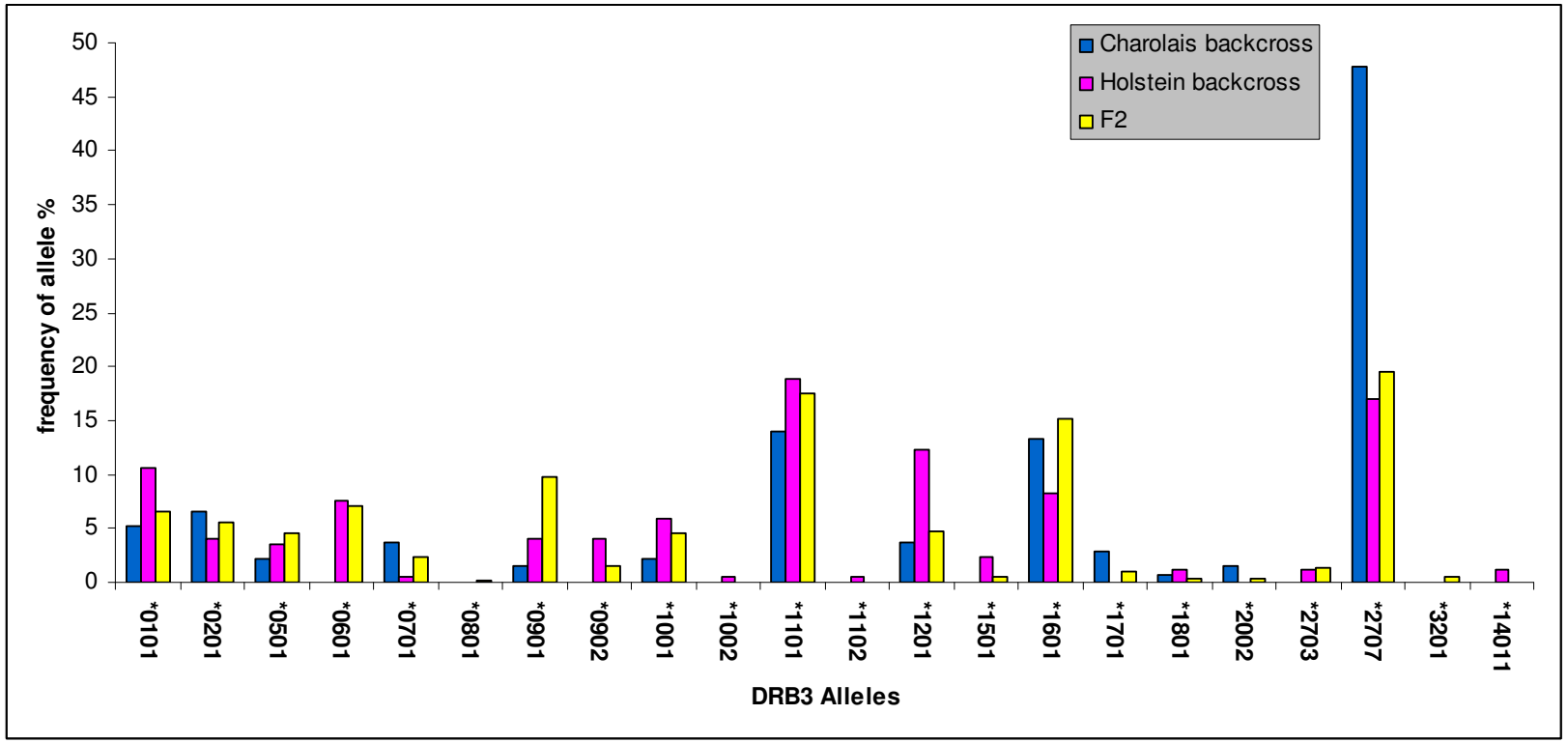


Figure 2.7 Allele frequencies in the Holstein backcross, Charolais backcross and F2 populations.

Each allele present in the RoBoGen herd has polymorphisms in the functional regions of the PBC. These functional regions have been defined as 'pockets' as they are the main point of interaction between the side chains of the bound peptides and the DR molecule (Sharif, Mallard, & Sargeant 2000). Table 2.1 shows the amino acids within the *DRB3*-encoded pockets for the alleles in the RoBoGen population.

	Pockets/Amino Acid Positions																	
	1	4				6	7						9					10
DRB3 allele	86	13	70	71	74	11	28	30	47	61	67	71	9	37	57	60	61	56
**0101*	M	S	E	K	E	S	D	Y	F	W	F	E	E	T	D	Y	W	Q
**0201*	V	S	R	A	A	S	D	Y	Y	W	I	R	E	F	D	Y	W	P
**0501*	V	S	R	K	N	H	D	Y	Y	W	I	R	E	Y	D	Y	W	P
**0601*	G	R	R	K	E	C	D	C	F	L	I	R	E	F	V	H	L	R
**0701*	V	R	E	R	E	C	D	C	F	W	F	E	E	F	V	Q	W	R
**0801*	V	S	D	E	S	A	D	Y	F	L	F	D	E	L	S	H	L	P
**0901*	G	S	R	K	N	S	E	S	Y	W	I	R	E	N	D	Y	W	P
**0902*	G	S	E	R	E	S	E	S	Y	W	I	E	E	N	D	Y	W	P
**1001*	G	S	R	A	A	S	D	Y	Y	C	F	R	E	Y	V	Y	C	R
**1002*	G	R	R	A	A	Y	D	Y	Y	C	F	R	E	Y	V	Y	C	P
**1101*	V	G	R	R	E	H	D	H	F	W	F	R	Q	Y	S	Y	W	P
**1102*	V	G	R	K	E	Y	D	H	F	W	F	R	E	Y	S	Y	W	P
**1201*	G	K	R	A	A	T	N	Y	Y	W	I	R	E	F	D	Y	W	P
**14011*	V	G	Q	K	E	H	D	H	F	W	F	Q	E	F	A	Q	W	P
**1501*	V	S	R	E	Y	S	D	Y	Y	L	T	R	E	F	V	Q	L	P
**1601*	G	K	E	K	A	T	D	Y	Y	W	F	E	E	F	D	Y	W	P
**1701*	G	S	R	E	Y	A	H	Y	F	W	I	R	E	Y	D	Y	W	P
**1801*	V	S	R	E	Y	A	H	Y	F	W	T	R	E	F	A	Q	W	P
**2002*	G	R	Q	R	A	C	D	Y	F	W	F	Q	E	R	D	Y	W	P
**2703*	G	R	E	R	E	Y	D	C	F	W	F	E	E	T	D	Y	W	P
**2707*	V	R	E	R	E	Y	D	C	F	W	F	E	E	T	D	Y	W	P
**3201*	G	R	R	E	Y	C	D	Y	F	W	I	R	E	Y	D	Y	W	P

Table 2.1 RoBoGen *DRB3* alleles and amino acids present in binding pockets.

A: arginine; C: cysteine; D: aspartic acid; E: glutamic acid; F: phenylalanine; G: glycine; H: histidine; K: lysine; L: leucine; M: methionine; N: asparagine; P: proline; Q: glutamine; R: arginine; S: serine; T: threonine; V: valine; W: tryptophan; Y: tyrosine.

2.5 Discussion

In order to assign functional importance to particular SNPs in polymorphic genes, it is clearly important to be able to accurately distinguish alleles. This is particularly relevant for the MHC loci as there are many polymorphic genes, most of which impact on the immune response to infectious pathogens and vaccines.

BoLA *DRB3* polymorphisms have in particular been associated with many diseases in cattle (Maillard, Martinez, & Bensaid 1996; Sharif et al. 1999; Garcia-Briones et al. 2000; Glass 2004). This gene is highly polymorphic and each allele contains many SNPs, unlike most other polymorphic genes. In order to determine which SNPs have functional importance in immune trait association studies, much greater accuracy can be achieved with exact sequence information.

The SBT technique described here has enabled the typing of 409 animals at the *DRB3* locus, with 22 different alleles successfully identified. Each of the 22 alleles has polymorphisms in the defined peptide binding pockets resulting in an amino acid change. This demonstrates that even small coding differences, e.g. 2 bp between **2707* and **2703* results in an amino acid change of glycine to valine in pocket 1, potentially have functional consequences.

In the RoBoGen herd there is a mixture of Holstein-Friesian and Charolais *DRB3* alleles. The Holstein breed has been extensively examined at this locus and the majority of known *DRB3* alleles have been described in Holstein-Friesian animals (http://www.projects.roslin.ac.uk/bola/drb3pcr.html). It was therefore interesting to see if the Charolais back-cross animals had any new, previously unidentified alleles and if the frequencies differed in Holstein back-cross and Charolais back-cross cattle,

as they are genetically distinct breeds (McKay et al. 2008) which have been exposed to different selection pressures, i.e. milk versus beef production. The Charolais had less variety in alleles, although this is most likely due to the experimental design. The **2707* allele seems to originate from the Charolais and is very similar to the Holstein **2703* allele, except for two base pairs. Even the 2 bp difference can have a functional impact (**Chapter 3** and **4**). However, both populations had similar alleles and other than the **2707* allele, no significant differences in allele frequency were noted. A more comprehensive survey of the original breeds would be required to draw definite conclusions about allele presence and frequency.

Distinguishing between highly polymorphic alleles is difficult and needs a high resolution sequencing approach. Older techniques such as RFLP-PCR resolved some of the alleles at the *DRB3* loci but it had limitations, as not all alleles can be detected. Other methods e.g. PCR-SSCP provide a higher resolution compared to RFLP-PCR and additionally allow the identification of new alleles, as has been shown for ovine MHC genes (Kostia et al. 1998).

SBT, however, is a powerful technique which allows the genotyping of large numbers of animals with relative speed and ease. The improvement in the SBT technique described in this chapter significantly reduces the time and extent of manual intervention required to generate accurate genotype information. It is possible to then use this information to look at associations between polymorphic alleles and immune traits, e.g. response to disease or vaccines. Another major advantage of the SBT technique is the possibility of finding *de novo* alleles.

Miltiadou et al. (2003) successfully implemented a sequence-based typing method for *DRB3* alleles in the RoBoGen herd. Nonetheless Miltiadou (2001) reported difficulties in accurately typing all animals due to problems towards the end of the hypervariable region. The previous method did not allow complete typing of this region because of the placement of the primer sets. However, this is overcome in this analysis by only using the outer set of primers, which allows the complete sequencing of exon 2 and therefore the unambiguous identification of all alleles.

Another difficulty with the original method was in deciphering the sequence after a deletion. In the RoBoGen herd, allele **0201* (frequency of 6%) has a 3 base pair deletion (bp 180, 181 and 182). The sequence trace post-deletion in a heterozygous animal has many more heterozygous peaks (Figure 2.8). The input files in Haplofinder contained *** at the deletion point which consequently did not allow for the identification of the **0201* allele. A change to the input file together with the increased quality compared to the gel sequencing method have made the allele identification process relatively straightforward.

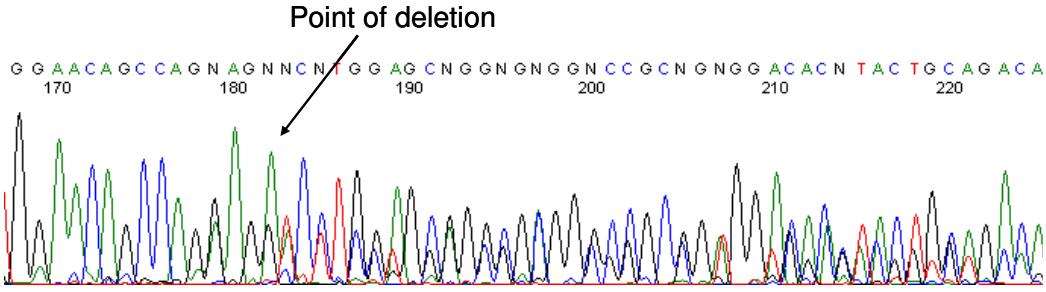


Figure 2.8 RoBoGen animal 119, *DRB3 *0201* and **1101* after 3 bp deletion. The sequence trace shows the point of deletion for the **0201* allele and after this point the majority of bases are heterozygous.

As most of the polymorphisms in the *DRB3* gene occur with the PBC encoding exon 2, it is interesting to see how this impacts upon the amino acid sequence. All of the alleles identified here have differences in the PBC, moreover they all have differences in the defined 'pockets' of the PBC. Therefore having the sequence data for the alleles allows a more in depth analyses of the interactions between the PBC and possible peptides.

The original typing of four animals was inaccurate, most likely due to the difficulties in base calling, especially in heterozygous animals, as a result of the gel-based Li-Cor system employed. Those which were different from the Miltiadou work were re-typed several times to confirm the new allele allocations (Table 2.2). The new allele allocations are likely to be correct as they achieved a quality score of at least 99% in the sequence traces. The increased length of the reads also means that the quality of the base calling towards the end of the sequence is increased. The increase in length of read has resulted in animal 44 being retyped as **1201* instead of **1202* (Table 2.2), a difference in a single base at the 3' end of the sequence.

Animal ID*	Sire	Miltiadou et al. alleles[1]		Baxter et al. alleles [2]	
16	R21	**1101*	**1801*	**1101*	**1101*
21	R02	**2707*	**2707*	**2707*	**0601*
31	R11	**2707*	**0902*	**2707*	**1101*
44	R11	**0501*	**1202*	**0501*	**1201*

Table 2.2 Comparison of original allele assignment with new allele assignments (Baxter et al. 2008). * Animal ID from Miltiadou et al. 2003.

[1] Miltiadou, Law & Russell 2003.

[2] Baxter et al. 2008

Other cattle MHC genes which are extremely likely to be important in the immune response against pathogens are the *DQ* genes. However, these genes have an extra layer of complexity as in some haplotypes the genes are duplicated. Therefore, using a SBT technique becomes harder, if not impossible, as an individual can have up to 4 different *DQA* and/or *DQB* alleles. An additional problem is that the alleles of the *DQ* genes are poorly defined. However, a group has now developed an SBT method for BoLA-*DQA*1, utilising the Haplofinder programme (Takeshima et al. 2007) to amplify only the *DQA1* genes, thereby reducing the complexity as these genes are not duplicated (although *DQA2* genes maybe present in the same haplotype). Whilst this method provides information on the *DQA1* alleles, it is limited as it does not type *DQA2, DQA3* or *DQA4* and it would not detect any duplication. In **Chapter 5** I will describe a microarray typing technique for *DQA* genes and a qPCR method for detecting duplication in the *DQ* genes.

Using improved sequence-based technology, it proved possible to accurately and rapidly type over 400 animals at the *DRB3* locus, providing detailed information of the SNPs present. The use of immune phenotypes together with results of the typing at *DRB3* will allow detailed investigation of associations between the polymorphisms and immune response, which is detailed later in this thesis (**Chapter 3** and **Chapter 4**). It seems likely that higher throughput sequencing technology will continue to develop and become even simpler and more affordable. In the future, it may be possible to harness this technology to investigate the association of *DRB3* and potentially other BoLA Class II genes, with larger phenotyped populations. Such data could then be used to quantify the extent to which MHC loci have an impact on

immune (and other commercial) traits. This information would enable breeders to make informed selection decisions.

3 Responses to Foot and mouth disease virus peptide and their associations with BoLA-*DRB3* in the RoBoGen herd

3.1 Introduction

3.1.1 Foot and mouth disease

Foot and mouth disease virus (FMDV) is a single-stranded, non-enveloped RNA aphthovirus of the *Picornaviridae* family, which affects cloven-hoofed animals. The virus particle contains 60 copies each of four structural proteins (VP1-4) which form an icosahedral capsid with seven serotypes (A, O, C, Asia 1 and SAT1-3) (reviewed by Grubman and Baxt (2004)) (Figure 3.1). The G-H loop is a major structural feature, which is highly immunogenic. The G-H loop has a highly conserved tripeptide arginine-glycine-aspartic acid (RGD) which mediates cell attachment (Burman et al. 2006). The high reproductive capacity of the virus, its ability to change its antigenic structure, persistence in the environment and its pathogenic consequences on productivity render it a serious threat to the beef and dairy industries in many countries (Thompson et al. 2002; Bates, Carpenter, & Thurmond 2003; reviewed by Grubman & Baxt (2004)).

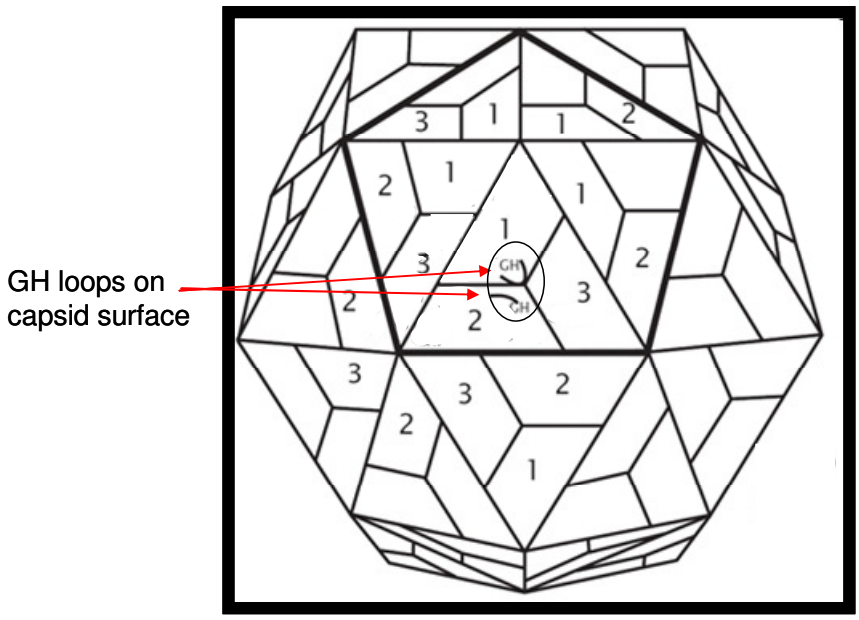


Figure 3.1 Diagram of FMDV capsid structure. There are three surface proteins, VP1, VP2, and VP3, labelled 1–3, respectively. The G-H loops which are highly immunogenic protrude from the capsid surface (Picture adapted from Frank, 2002).

FMDV is highly contagious and infection results in high morbidity especially in high producing cattle breeds, and can be fatal in immature animals (Kitching 2002). Recent outbreaks of FMD in Taiwan, South Korea and United Kingdom resulted in the slaughter of millions of animals and huge economic losses, due to restrictions on export of animals and meat and dairy products (Davies 2002). The management and control of the disease is therefore a major concern worldwide. In FMDV endemic regions, such as Africa, Asia and South America, control measures involve regular vaccination with inactivated virus vaccines (Sobrino et al. 2001; Anonymous 2003). These vaccines are not used in FMDV-free states such as the US and the EU, mainly because the benefits of trade in FMDV certified free products are considered to

outweigh the increased risk to susceptible livestock populations and costs of vaccination as a preventative measure. Additionally, in the UK, vaccination against FMDV has never been implemented and vaccination to control the two UK outbreaks was ruled out, possibly because of ideological doctrine as well as ensuing trade restrictions (Sutmoller et al. 2003). In the past, the use of inactivated viral vaccines made it difficult to distinguish vaccinated from infected animals (DIVA), but vaccines prepared from purified components do not contain, and therefore do not induce, antibodies to non-structural proteins (Sutmoller et al. 2003). In compliance with international law, outbreaks are controlled in FMDV-free states by a 'stamping-out' policy of quarantine and slaughter. However, following recent outbreaks recommendations have been made which may result in vaccination becoming the preferred option for controlling future outbreaks (The Royal Society 2002; Paton, Sumption & Charleston 2009). Unfortunately the commercially available vaccines have low efficacy, requiring regular boosts (Grubman & Baxt 2004). Biosecurity and biocontainment remain additional concerns, particularly as global trade and movement of livestock is likely to increase, and in the EU, countries that are adjacent to countries outside the EU that are not FMDV-free would appear especially at risk (Sutmoller et al. 2003). Additionally the accidental release of the virus during vaccine preparation and storage has been the origin of several FMD outbreaks, although with newer methods of inactivation this is a lesser concern (Sutmoller et al. 2003).

Alternative strategies for vaccine design are therefore needed such as, synthetic vaccines, which have the potential to be used in emergency situations to supplement current control measures, or as a replacement for the present vaccines in endemic

countries (Taboga et al. 1997). However, the design of a synthetic FMDV vaccine that elicits strong and long-lasting protection has proved difficult, although Wang et al. (2002) reported near complete protection of swine against challenge infection with FMDV after vaccination with a synthetic peptide.

The design of effective vaccines requires the identification of epitopes capable of inducing uniform strong and long-lasting immune protection in an outbred population. Furthermore, activation of humoral and cell-mediated immune responses is required for effective FMDV vaccine protection (Sobrino et al. 2001). Protection against FMDV has been associated with a high serum neutralising titre (SNT), dominated by immunoglobulin G1 (IgG1) and immunoglobulin G2 (IgG2) antibody subclasses (Collen, Dimarchi & Doel 1991; Sadir et al. 1999). The induction and maintenance of immunological memory is T cell dependent, thus it was considered that a strong $CD4^+$ T cell response must be necessary for an efficacious FMD vaccine (Collen, Dimarchi, & Doel 1991). Interestingly, more recent work has suggested that resolution of an acute primary FMDV infection may be T cell-independent and the repeating nature of the capsid structure may enable T-independent antibody class switching to occur (Juleff et al. 2009). Nonetheless it seems probable that a protective vaccine would need to induce the development of both T cell and antibody memory responses. In addition, $CD8^+$ T cells may play a role in protection (Collen 1994; Childerstone et al. 1999; Sobrino et al. 2001; Guzman et al. 2008).

Although a humoral response is generally accepted as the main protective mechanism against FMDV, the correlation between the levels of neutralising antibody and protection are not always apparent, especially in the case of peptide or

subunit vaccines, and it is clear that other factors play a role, including both innate (Summerfield et al. 2009) and different aspects of adaptive immunity (Collen 1994). In terms of the latter, about which more is known, FMDV IgG1 titres were shown to correlate with protection (Capozzo et al. 1997), and IgG1 is an antibody isotype in cattle associated with a Th2 response (Estes & Brown 2002). In contrast, another study has shown that an interferon-γ (IFN-γ) response also correlates with protection (Parida et al. 2006), which makes sense as this T cell-derived cytokine can inhibit FMDV replication *in vitro* (Zhang et al. 2002). T cell-derived interleukin (IL)-2 also plays a role in protection (Amadori et al. 1992). Although T cells responding to the inactivated viral vaccine produced mRNA for IFN-γ and not IL-4, (Van Lierop et al. 1995), it seems likely that both Th1 and Th2 responses play a role in protection against FMDV.

The major neutralising antibody epitopes have been identified as the amino acids that make up a prominent G-H loop structure of the viral capsid protein, VP1, together with the VP1 C-terminal residues which are contiguous in the native virion (Dimarchi et al. 1986). A synthetic vaccine against FMDV would therefore be required to include a region that mimics this loop structure. In addition to this, a synthetic peptide would need to include regions identified as potential T cell epitopes, for example the RGD integrin binding motif and the lysine-valine-alanine-arginine (KVAR) T cell motif (Rothbard & Taylor 1988). A 40-mer peptide (FMDV15) representing the G-H loop region and containing both the RGD and KVAR motifs elicited high levels of neutralising antibody and protection of cattle against FMDV (Dimarchi et al. 1986). The 40-mer peptide has two cysteines at the N-terminus and a proline-cysteine-glycine (PCG) at the C-terminus with a proline-

proline-serine (PPS) spacer to mimic the GH loop structure. Nevertheless, high-dose inoculation with FMDV15 was required, and the levels of SNT obtained did not correlate with protection (Dimarchi et al. 1986). Subsequent studies following improvements in peptide design and vaccine delivery have led to more consistent protection against FMDV (Cubillos et al. 2008; Greenwood et al. 2008).

In the move towards more refined vaccines, variable immune responses and hence variable protective efficacy are beginning to be recognised as problems, with some individuals remaining as non-responders even after repeated vaccinations (Poland, Ovsyannikova, & Jacobson 2008). These differences are partly determined by environmental factors, such as the presence of maternal antibodies, poor nutrition or concurrent infections, and partly by the genetic background of the vaccine recipients. The genetic differences in vaccine-induced immune responses may be explained by the diversity of genes expressed in the immune system, such as the toll-like receptors and cytokines (Poland et al. 2007). However, the role of host genetics, which may underlie variable immune response to vaccination has not been thoroughly explored (reviewed by Glass (2004)). The effect of host genetic variation on vaccine response is most likely mediated through polymorphisms in many genes. There has been a renewed focus on the major histocompatibility complex (MHC), due to the important role MHC-encoded proteins play in the host immune response, particularly to epitope-based vaccines (Sette & Fikes 2003). Studies also suggest that where genetics is shown to play a role in the phenotypic variation in response to vaccines and pathogens, a significant proportion can be attributed to MHC genes (Glass 2004; Poland et al. 2007). Although there are QTLs which map to other regions within the genome for the response to the FMDV peptide (Leach et al. 2010). The limited

repertoire of epitopes in peptide/synthetic vaccines compared to the native organisms increases the importance of the interaction between the vaccine epitopes and the MHC molecules in generating an effective homogenous adaptive immune response in outbred populations.

3.1.2 Cattle MHC and FMDV

The MHC and bovine MHC in particular are described in **Chapter 1**. This section concentrates on the relevance of the MHC to variation in the immune response to FMDV and antigen derived from it.

MHC restriction may play a particularly relevant role in protective responses to FMDV, because unlike other members of the Picornaviridae family, the major protective mechanism, in the form of virus-neutralising antibodies, is focused on the prominent G-H peptide loop structure on its surface. Two earlier studies have shown that *DRB3* alleles influenced the immune response induced by FMDV peptides containing this neutralising antibody site (Glass, Oliver, & Spooner 1991; Glass & Millar 1994), with several of the alleles showing suggestive associations with protection against viral challenge (Garcia-Briones et al. 2000). Glass and colleagues (2000) have also shown that an FMDV peptide containing the neutralising epitope induced T cells restricted by both BoLA DRB3 and DQ molecules. A number of papers have also described associations of *BoLA DRB3* with response to other epitopes in the native virus and also inactivated viral vaccines (Collen, Dimarchi, & Doel 1991; Van Lierop et al. 1995; Gerner et al. 2007). Recently, Gerner et al. (2009) identified a putative DQ epitope in VP4. However, the role of *BoLA DQ* alleles in variation in immune responsiveness to FMDV is unclear. Additionally,

infection with native virus and an inactivated viral vaccine generated FMDV-specific BoLA Class I-restricted $CD8^+$ T cells (Guzman et al. 2008). BoLA Class I is also complex, with different numbers of BoLA Class I molecules encoded depending on haplotype (Ellis 2004). However again, what role, if any, BoLA Class I alleles might play in variation in the protective immune response to FMDV is unknown.

Thus the available evidence would suggest that an effective vaccine to FMDV has to interact with BoLA molecules, include the major epitope of the component of FMDV recognised by the neutralising antibody, and take account of *BoLA* polymorphism, especially *BoLA DRB3*.

The binding pockets (described in **Chapter 1**) will play an important role in the binding of the peptide for presentation. In human HLA-DR molecules, pocket 4 has been found to be important for the binding of peptide (Fu et al. 1995). This may be due to pocket 4 being located in the centre of the PBC. In addition it has been reported in cattle that the pocket 4 motif can have a significant effect on the immune response (Sharif, Mallard, & Sargeant 2000; Sitte et al. 2002). Pockets and motifs other than pocket 4 also affect the immune response in both humans (Zerva et al. 1996) and cattle (Xu et al. 1993; Maillard, Martinez, & Bensaid 1996; Sitte et al. 2002; Zavala-Ruiz et al. 2004). Furthermore, the binding affinity of FMDV peptides to different allelic forms of purified BoLA-DRB3 correlates with T cell proliferation (Haghparast et al. 2000).

3.2 Aim and Hypothesis

The aim of this chapter was, therefore, to explore the associations between the BoLA-*DRB3* alleles and the immune response to FMDV15, a 40-mer peptide derived

from FMDV Serotype 0 containing neutralising epitopes, which was the peptide described by DiMarchi et al (1986). The hypothesis was that it would be possible to find significant associations which would provide information on the role of BoLA-*DRB3* in immune responsiveness in cattle.

3.3 Experimental design

Specifically, a large crossbred cattle population was immunised with FMDV15 and IgG1, IgG2, T cell and IFN-γ responses were measured. The cattle population (RoBoGen herd) were part of a larger study on the role of genetics in performance and immune traits. They were naive to FMDV and thus provided a unique opportunity to investigate the role of MHC in the primary and secondary humoral and cellular responses to an antigen to which the animals had not previously been exposed. The improvements in *BoLA-DRB3* typing of exon 2, which encodes the PBC containing the majority of the polymorphic sites in the DR molecule (Baxter et al, 2008; see **Chapter 2**), made feasible the genotyping of a large number of animals. This in turn enabled the analysis of the impact of specific alleles, as well as the polymorphisms in individual amino acid positions and the anchor "pockets" of the PBC, on the response to FMDV15 to be determined. This was accomplished through a mixed model statistical analysis of the associations between the alleles of *BoLA-DRB3* and the phenotypic immune measurements. In addition, a preliminary exploration of the DR protein was studied through 3D modelling. Part of this chapter has been published, specifically the antibody response data (Baxter et al. 2009).

3.4 Materials and methods

3.4.1 Animals

The study population was a second generation cross between Charolais and Holstein cattle. The herd was bred from four pure-bred Charolais sires and eight F_1 Charolais-Holstein sires. The 12 sires were used to produce a second generation cross composed of inter-crossed (F_1 x F_1 n=123), and backcross (Charolais x F_1 n=31 and Holstein x F_1 n=43) animals, giving a total of 197 female animals that were included in this study. Blood samples were collected across three separate cohorts from 1999 to 2001. Immunisation age ranged from 469 to 609 days.

3.4.2 Immunisation and sampling

The FMDV15 peptide represents a fragment of the VP1 capsid protein that includes amino acids 200-213 and 140-158, coupled by a Pro-Pro-Ser spacer together with a dicysteine residue at the N terminus and Pro-Cys-Gly at the C terminus (Dimarchi et al. 1986) (Figure 3.2). The peptide was prepared by Dr A. Douglas, Veterinary Services Division, Agri-Food and Biosciences Institute, Belfast, on an ABI 431A peptide synthesiser using FMOC chemistry. Following deprotection and cleavage, it was purified by preparative reverse phase HPLC (Beckman System Gold HPLC using a Phenomenex Luna C18 column).

Figure 3.2 FMDV15 40-mer amino acid sequence derived from VP1 capsid protein. The red letters are the spacer amino acids and the N-terminus and C-terminus additions. The green letters are the identified B and T cells epitopes.

Animals were immunised subcutaneously with FMDV15 peptide (1 mg/animal) emulsified in Freund's incomplete adjuvant, with a further immunisation (100 µg/animal) 6 weeks later essentially as described previously (Dimarchi et al. 1986; Glass et al. 1991). To determine the animals' immune response to vaccination, blood samples were collected by jugular venipuncture into serum tubes for antibody analysis (Greiner Bio-one, UK), and for the T cell and IFN-γ analysis, blood was collected aseptically into heparin tubes (Greiner Bio-one). Samples were taken at vaccination (day 0) and 1, 2, 4, 8 and 10 weeks later. Each cohort was immunised and sampled over three consecutive years. All animals were clinically normal. All experimental protocols were authorised under the UK Animals (Scientific Procedures) Act, 1986.

3.4.3 ELISA for detection of FMDV15-specific IgG1 and IgG2

The enzyme-linked immunosorbent assay (ELISA) for the detection of FMDV15-specfic IgG1 and IgG2 is fully described in Baxter et al. 2009.

3.4.4 Cell proliferation assay

The proliferation assay was carried out essentially as previously described (Glass et al. 1991) with the exception that whole blood was used (Appendix C.2). Preliminary experiments confirmed that the major proliferating population was T cells (results not shown). The stimulation index (SI) was calculated (ratio of cpm after culture with either FMDV peptide or ConA divided by the cpm of control cultures) and used in further analyses.

3.4.5 IFN-γ assay

IFN-γ release by whole blood cells *in vitro* were determined by Enzyme Amplified Sensitivity Immuno Assay (EASIA) (Catalogue number KAC123, Biosource International, now Invitrogen) using whole blood in heparin, essentially according to the manufacturer's guidelines, except that FMDV peptide and ConA were used as the test antigens, and ovine recombinant (r) IFN-γ (a kind gift from Professor Gary Entrican, Moredun Research Institute, UK) was used to determine IFN-γ concentrations (Appendix C.2).

The negative control values for the IFN-γ levels were then subtracted from the results obtained for FMDV15 and ConA and these values were used in the statistical tests below. Due to a late decision to include a positive control there are no ConA results for cohort 1.

All immunisations and assays were carried out by technical and farm staff at The Roslin Institute.

3.4.6 Sequence-based typing method of *DRB3*

Exon 2 of the *DRB3* gene was amplified from genomic DNA from all 197 animals. The resulting amplicons were sequenced by BigDye (Applied Biosystems) sequencing reaction as previously described (see **Chapter 2**; Baxter et al. 2008). *DRB3* genotypes were allocated to all animals using Haplofinder (http://www.bioinformatics.roslin.ac.uk/haplofinder/haplofinder.py).

The amino acids at pocket positions were determined from the nucleotide sequences of the alleles (BioEdit Hall 1999) and the pocket positions (Sharif, Mallard & Wilkie 2003).

3.4.7 Statistical analysis

Mixed linear models, such as residual maximal likelihood (REML) (Patterson & Thompson 1971), were considered to be the most appropriate for analysis of the effects of *DRB3* alleles on antibody and T cell response to FMDV15. A REML model was chosen as it can account for a number of covariates and factors that have an impact on the traits whilst accounting for the relationship between animals in the form of a random effect of dam. The fixed effects in the model with appropriate degrees of freedom (d.f.) were breed cross (Charolais Backcross (CB), Holstein Backcross (HB), F2 generation, (2d.f.)) and Cohort (1999, 2000, 2001 (2d.f.)). The effect of MHC allele or amino acid at certain positions was examined by contrasting each analysed variant with all other possible variants (0: no copies, 1: a single copy or 2: two copies (2d.f.)). Immunisation age was defined as a continuous variate and calf dam was included as a random effect. Thus the linear model was:

$$y_{ijkl} = l_i + c_j + u_k + MHC_{ijkl} + a_{ijkl} + e_{ijkl}$$

where: y_{ijkl} is the observed phenotypic trait; l_i fixed effect of breed-cross (CB, HB or F2); c_j fixed effect of the cohort (years 1, 2, or 3); linear regression of the MHC allele/amino acid (0, 1 or 2 copies); u_{ijkl} random effect of the dam; a_{ijkl} covariate

effect of the age of the animals at time of vaccination, e_{ijkl} is the residual error. All statistical analysis was performed using Genstat with the significance of fixed effects, such as MHC variant being assessed by a Wald test (Genstat 9.0v VSN international). If MHC variants were significant (at least $p<0.05$), then further analysis was conducted by generating predicted means and conducting a Student's t-test using pair-wise comparisons. All statistical analysis was performed using Genstat.

3.4.8 IgG1 and IgG2

The IgG1 and IgG2 concentration data were normalised by Log_e transformation to obtain a normal distribution.

3.4.9 T cell proliferation

The T cell assay had three concentrations of FMDV15 peptide (0.1, 1 and 2 µg/ml) and three concentrations of the positive control, ConA (0.25, 2.5 and 10 µg/ml). The data were analysed using log_e transformed stimulation index (SI). However, there were difficulties analysing this data set as there was a low response after exposure to the FMDV peptide, with some of the control results having a higher rate of proliferation than the test FMDV results. Each of the transformed data sets was used in the REML model (see above). Only the results from the FMDV 2 µg/ml and ConA 10 µg/ml are shown, but the significant alleles remained significant across concentrations.

3.4.10 IFN-γ

The IFN-γ dataset had many non-responders (animals which did not make IFN-γ above 0 after control levels have been subtracted) at each time point (88, 31, 37 and 56 animals at weeks 0, 4, 8 and 10 respectively), including 8 animals which did not make any detectable IFN-γ response at any of the time points. It would be expected that as the animals were naïve to the FMDV15 peptide all would not respond at week 0 (see discussion). Due to the high numbers of non-responders, transforming the data through Log_e transformation and square-root transformation did not normalise the distribution. Thus, to analyse this dataset a generalised linear mixed model (GLIMM) approach with a binomial distribution was chosen.

A generalised mixed linear model was implemented:

$$y_{ijkl} = l_i + c_j + u_k + MHC_{ijkl} + a_{ijkl} + e_{ijkl}$$

where: y_{ijkl} is the observed IFN-γ level either 0 for non response or 1 for response; l_i fixed effect of breed-cross (CB, HB or F2); c_j fixed effect of the cohort (years 1, 2, or 3); linear regression of the MHC allele/amino acid (0, 1 or 2 copies); u_{ijkl} random effect of the dam; a_{ijkl} covariate effect of the age of the animals at time of vaccination, e_{ijkl} is the residual error. All statistical analysis was performed using Genstat with the significance of fixed effects, such as MHC variant, being assessed by a Wald test (Genstat 11.0v VSN international).

3.4.11 Area under the curve

To examine the overall response across time points to the FMDV15 peptide, the area under the curve (AUC) was calculated using the trapezoid rule (Appendix C.1) for

IgG1 and IgG2, and was used in the REML model. Due to the structure of the IFN-γ and T cell dataset the AUC was not calculated.

3.4.12 3D modelling of *DRB3*1001* and **1601*

Overall the results presented in this chapter showed that the DRB3 pocket positions were highly significantly associated with the magnitude of the ensuing immune response. Thus it would seem highly probable that this is due to the binding affinity of peptide to the DRB3 molecule. It was decided that the use of 3D modelling might provide more insight into the structure of the BoLA DR pockets. The modelling of both the BoLA-DR molecules represents very preliminary data. Two alleles were chosen for the modelling, DRB3**1601* and **1001*, as they were shown in the statistical analysis to be associated with lower and higher IgG1 and IgG2 responses respectively. As there is no crystal structure available for bovine DRB3, the modelling was based on a human equivalent. The modelling of the MHC molecules was undertaken at the Roslin Institute by Dr. Zen Lu.

The initial step in the modelling was to identify the most appropriate template. In order to complete this, the DRB3*1601 sequence was submitted to the Template Identification tool of the Swiss-Model web server (http://swissmodel.expasy.org/workspace/index.php?func=tools_targetidentification1) and also BLASTed against the PDB database (http://www.pdb.org/pdb/search/advSearch.do). It was found, from aligning all the various DRB3 alleles from all mammalian DRB3 sequences available using the program ClustalW (version 1.83), that the bovine allele *1601 is most closely related

to the human leukocyte antigen DR52a (HLA-DRA, *DRB3*0101*) (PDB accession number: 2Q6W, http://www.pdb.org/pdb/explore/explore.do?structureId=2Q6W). The HLA β-chain of 2Q6W shares about 84% sequence identity with BoLA *DRB3*1601*.

The confidence of 2Q6W being the best template is further enhanced by the fact that when DRB3_1601 was submitted to the Swiss-Model for automated model prediction (http://swissmodel.expasy.org/), the server also picked the same structure to model the **1601* allele.

Sequences of DRB3*1601 and 2Q6W were next aligned against each other and resubmitted to the Swiss-Model server for alignment-mode modelling. The modelled structure together with the template were downloaded and imported into the Swiss PdbViewer (version 4.0) where several rounds of iterative Magic Fit and Energy Minimization (tools available in Swiss PdbViewer) were applied to get a more reliable structure for DRB3*1601. The template structure was removed before saving the final BoLA DRB3*1601 structure. This improved DRB3*1601 was validated using the Protein Structure & Model Assessment Tools of the Swiss-Model server. The Anolea atomic mean force potential and Stereochemical quality Procheck (Laskowski et al. 1993) of the modelled structure were calculated to check for any anomaly.

The DRA α chain was also modelled by following the steps above. The template used was the crystal structure of the DR alpha chain of human HLA Class II histocompatibility antigen (PDB accession number: 1JWU, http://www.pdb.org/pdb/explore/explore.do?structureId=1JWU).

To construct the functional dimerised unit of the bovine Class II histocompatibility antigen, the two modelled β- and α-chains of DRB3*1601 and DRA were merged using the 2Q6W (complete human DR molecule) as the template: The structure 2Q6W was loaded into the program GRASP2 (http://wiki.c2b2.columbia.edu/honiglab_public/index.php/Software:GRASP2). The modelled BoLA DRB3*1601 and BoLA DRA were next imported into the Swiss PdbViewer. The merged bovine DRA and DRB3*1601 structures were finally saved as a single dimer and validated as above. The same process was carried out for the modelling of DR molecule with the *DRB3*1001* allele.

3.5 Results

3.5.1 FMDV15-specific IgG1 and IgG2

The dose and time course intervals were chosen based on earlier studies that had characterised the response to the FMDV15 peptide in Holstein cattle (Glass et al. 1991). It was expected that the majority of animals would respond. The FMDV-specific IgG1 and IgG2 concentrations were measured by ELISA in all 197 animals. The levels of both IgG isotypes were not significantly different from zero at weeks 0 and 1. From week 2 onwards, both IgG1 and IgG2 responses showed a high degree of variation among individuals in response to immunisation with the FMDV peptide (Figure 3.3). The mean IgG1 concentrations increased with time and at weeks 2, 4, 8 and 10 were 69.6, 166.0, 289.6 and 175.0 µg/ml, respectively (Figure 3.2a). The AUC had a mean of 1648 µg/ml and a range of 0.068-642.0 µg/ml.

IgG2 levels were lower than the IgG1 levels in the majority of animals, but still showed a consistent response to the FMDV15 peptide. The mean FMDV15-specific

IgG2 concentrations at weeks 2, 4, 8 and 10 were 5, 13, 23.97 and 23.30 µg/ml respectively (Figure 3.3b). The AUC had a mean of 101 µg/ml and range of 0-372 µg/ml. The majority of the animals had responded by week 2. However there were a small number of animals that had a low response even after week 4. Fifteen of the 197 animals did not make any detectable IgG2 response to the FMDV15 peptide at any time point.

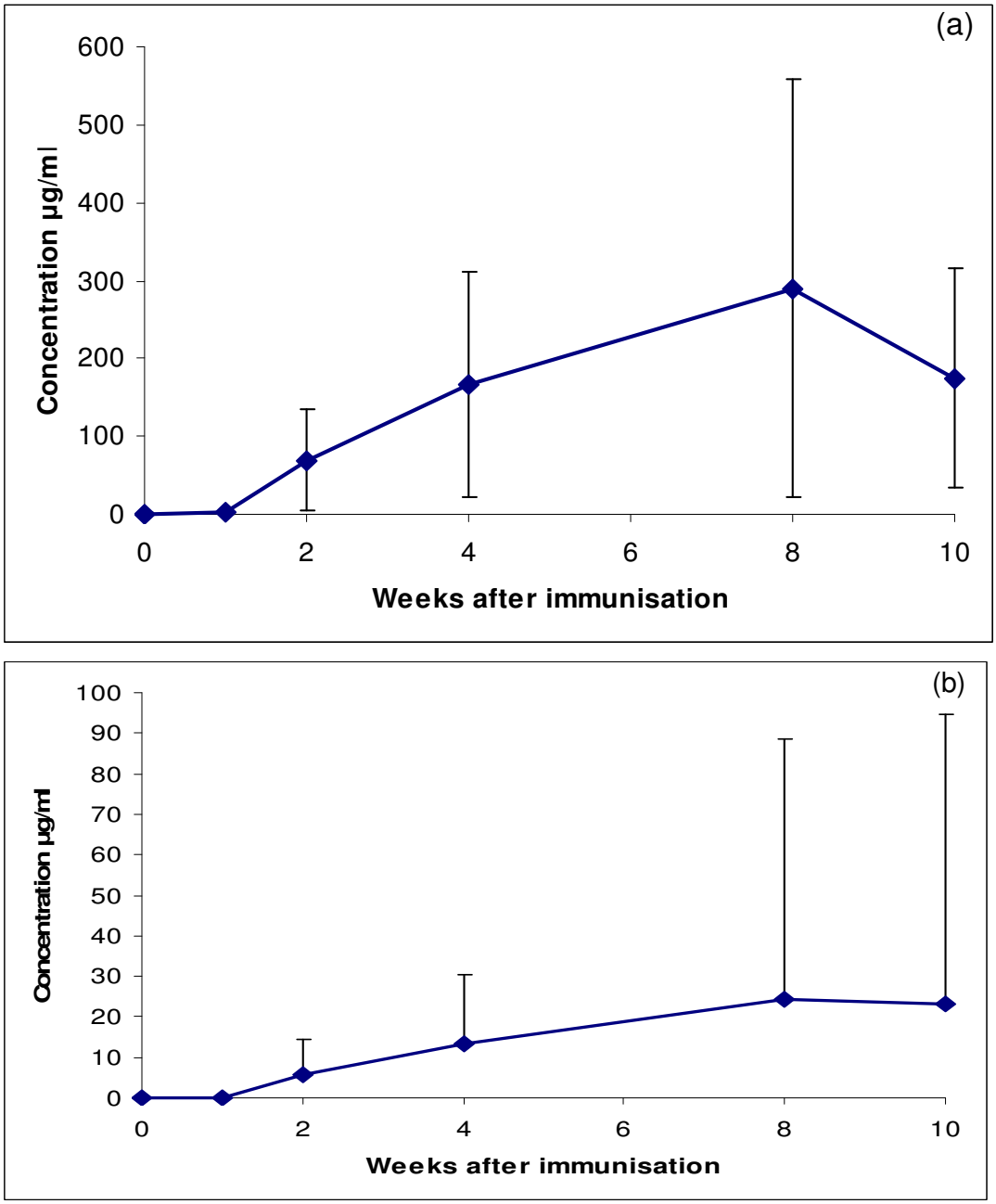


Figure 3.3 Antibody responses after immunisation with FMDV15 peptide. Anti-FMDV15 IgG1 (a) and anti-FMDV15 IgG2 (b). Mean concentration (± SD).

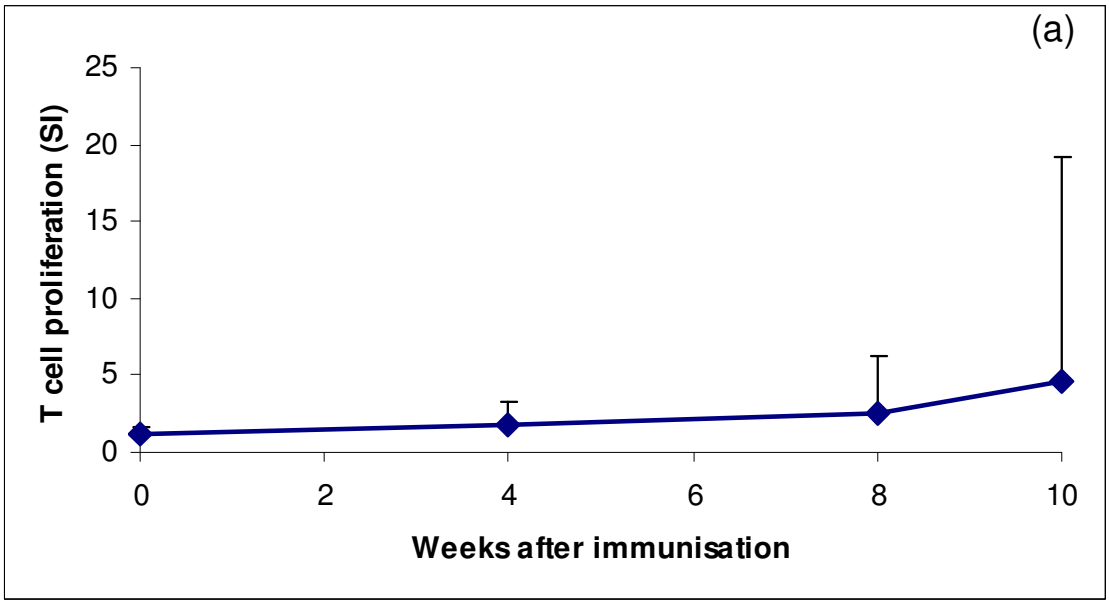
3.5.2 T cell proliferation to FMDV15 and ConA

3.5.2.1 FMDV15

The analysis for the T cell proliferation is only shown at the highest concentration for both FMDV15 and ConA for simplicity (the lower concentrations had similar graph profiles). Proliferation of T cells after immunisation with the FMDV15 peptide showed a SI increase from 1.13 to 4.58 (week 0 to week 10) with a high degree of animal to animal variation observed at weeks 8 and 10 (Figure 3.4a).

3.5.2.2 ConA

The mean T cell proliferation for ConA was higher than for FMDV15 with weeks 0, 4, 8 and 10 mean SI values of 13.85, 17.67, 13.35, and 18.76 respectively. The T cell proliferation after exposure to ConA was virtually equal across all weeks. However high animal to animal variation was observed across all time points (Figure 3.4b).



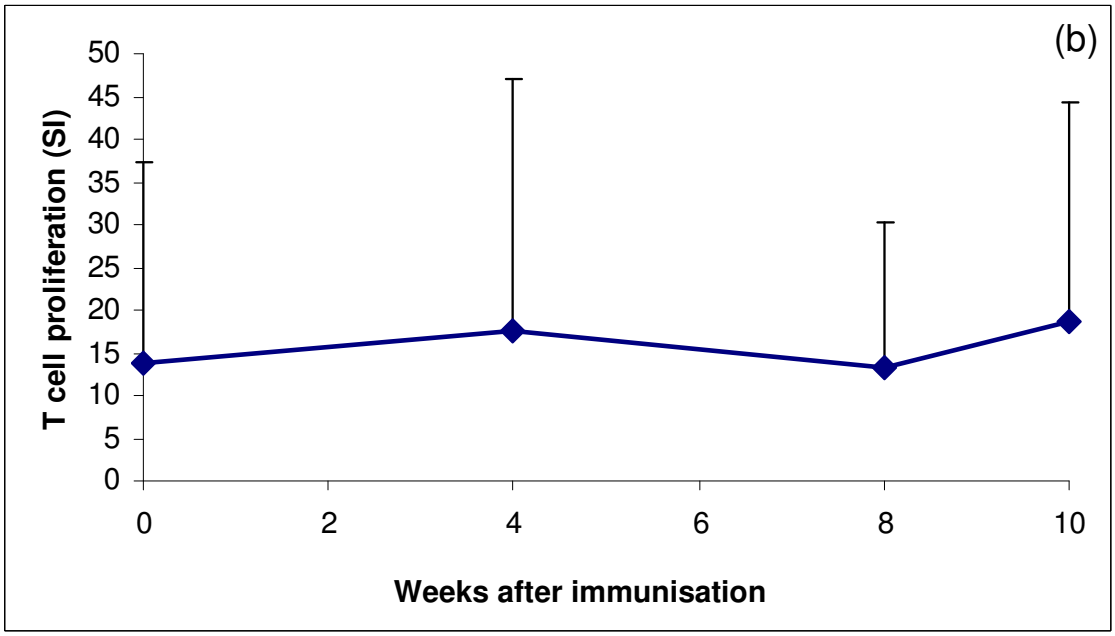


Figure 3.4 T cell proliferation after immunisation with 2 µg/ml FMDV15 (a) and 10 µg/ml ConA (b). Mean SI across time points ± SD.

3.5.3 IFN-γ response to FMDV15 and ConA

The results from the IFN-γ assay showed many animals failing to produce any detectable IFN-γ at each time point (Table 3.1). In addition there were high levels of animal to animal variation (Figure 3.5a and b).

	FMDV15 (week)			
	0	**4**	**8**	**10**
nonresponders (%)	65	21	24	36
	ConA (week)			
	0	**4**	**8**	**10**
nonresponders (%)	30	29	23	41

Table 3.1 Percentages of nonresponders IFN-γ to FMDV15 and ConA at each week following immunisation.

3.5.3.1 FMDV15

The mean values of the IFN-γ response to FMDV15 were 106, 622, 714 and 435 pg/ml for weeks 0, 4, 8 and 10 respectively (Figure 3.5a). The greatest variation in levels of IFN-γ in response to the FMDV peptide was at the peak week (week 8) with a range of 0-1665 pg/ml.

3.5.3.2 ConA

ConA was the positive control for the production of IFN-γ. The levels of IFN-γ with a concentration of ConA at 10μg were 100, 219, 761 and 189 pg/ml for weeks 0, 4, 8 and 10 respectively (Figure 3.5b). Unfortunately weeks 0 and week 4 for cohort 1 do not have any ConA results. The greatest variation was seen at week 8 with a range of

0-1887 pg/ml with 41 animals failing to make any detectable IFN-γ response. Five animals did not make any detectable IFN-γ response to either the FMDV15 or the ConA at any time point. The IFN-γ FMDV15 levels were positively correlated with IFN-γ ConA levels and this was highly significant ($p<0.001$).

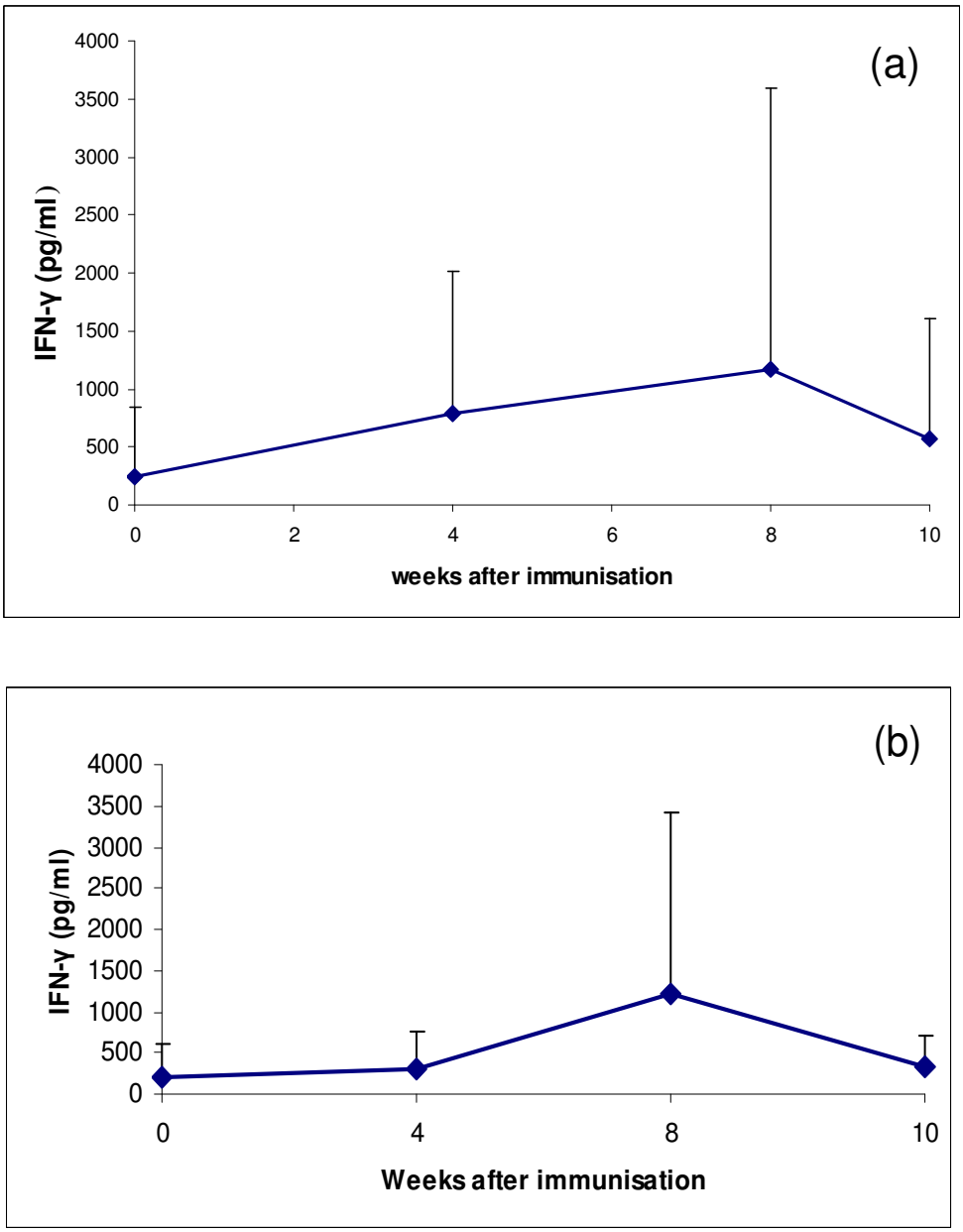


Figure 3.5 T cell derived IFN-γ levels after immunisation with FMDV15 (a) and ConA (b). Mean concentration (pg/ml) across time points ± SD.

3.5.4 Alleles within the herd

All 197 female animals were genotyped at the *DRB3* locus using a sequence-based typing method (Baxter et al. 2008). However, 19 animals were excluded due to poor quality genomic DNA that resulted in poor sequence data. A total of 18 different *DRB3* alleles were identified in these animals (Table 3.2). The allele with the highest frequency was *DRB3*2707* (20%) primarily due to the crossbred herd structure as 3 of the 4 founding sires had *DRB3*2707*. Alleles **1101* (16%) and **1601* (10%) were the second and third most frequent within the population. Six alleles (**1501, *1701, *1801, *2002, *2703* and **3201*) were represented six times or less in the herd and were excluded from the REML analysis presented in this chapter (Appendix C.2, C.3).

3.5.5 Association of *DRB3* alleles with the IgG1and IgG2 response to FMDV15 peptide

3.5.5.1 IgG1

IgG1 mean, median and upper and lower quartile levels for week 8 (peak week) were examined for each of the *DRB3* alleles present within the herd (Table 3.2). The alleles were ranked according to the mean IgG values. The IgG1 levels were positively skewed as indicated by the median and quartile values. *DRB3 *0901* allele was associated with the highest mean value, whereas *DRB3*1801* allele was associated with the lowest value. The *DRB3* alleles which showed a significant association with IgG1 levels ($p<0.05$) for more than a week are shown in Table 3.3 and all other allele results are shown in Appendix C.2. The top ranked allele,

*DRB3*0901* (Table 3.2), was significantly associated with the IgG1 response at both weeks 2 and 8 (Table 3.3) ($p<0.05$ for both time points). *DRB3*1001,* which was ranked 8th (Table 3.2) and also significantly correlated with the IgG1 responses ($p<0.05$), had a significantly positive effect across the time points (Figure 3.6a). The most frequent allele in the herd was *DRB3*2707,* with a frequency of 20%, and was associated with the 3rd lowest mean response, while the 3rd most frequent allele *DRB3*1601* also gave a low scoring mean value (Table 3.2), ranked 5th from last. Both (**2707* and **1601*) of these alleles were highly significantly associated with the IgG1 response at several time points (Table 3.3). The presence of *DRB3*1601* allele was correlated with lower IgG1 levels (Figure 3.6b). Animals positive for 2 copies of **2707* had a significantly lower IgG1 response ($p<0.001$) to the FMDV15 peptide than animals which did not have a **2707* allele (Figure 3.6c). The predicted means values used for the student's t test were 0 copies of **2707* and 2 copies of **2707*. Two further alleles, *DRB3*0701* and *DRB3*0902*, were associated with higher and lower responses respectively (Table 3.2) although both alleles were only significant at a single time point, albeit that in the case of *DRB3*0701* the total response (AUC) was also significant (Appendix C.2).

DRB3 allele	Frequency*	Number of animals with allele	FMDV15 specific IgG1 Levels (µg/ml)			
			Mean	Median	Lower quartile Q1	Upper quartile Q2
**0901*	0.06	22	468.0	412.0	200.0	735.0
**0701*	0.02	8	422.8	209.0	150.0	806.0
**2002*	0.01	4	398.9	421.0	323.0	474.7
**2703*	0.01	3	385.8	430.7	179.3	581.1
**1101*	0.16	52	379.7	307.5	109.5	533.5
**1201*	0.06	22	355.2	280.0	211.0	464.0
**3201*	0.01	2	349.4	349.4	208.4	490.4
**1001*	0.05	18	332.1	284.0	110.0	494.0
**0501*	0.04	13	311.3	236.0	118.5	412.8
**0601*	0.06	18	308.9	258.0	56.0	394.0
**1501*	0.02	6	256.8	132.3	94.3	193.0
**0201*	0.07	25	255.2	211.0	81.5	335.0
**0101*	0.06	21	241.5	138.0	79.0	315.2
**1601*	0.10	37	239.6	166.3	82.2	341.4
**0902*	0.04	13	206.2	195.0	59.2	297.2
**2707*	0.20	68	205.1	140.4	67.4	279.8
**1701*	0.01	2	116.0	116.0	11.0	221.0
**1801*	0.01	4	111.6	89.0	69.5	153.6
Overall	1.0	178	289.0	213.8	102.1	405.2

Table 3.2 *DRB3* alleles in the population: mean, median, upper and lower quartiles at peak week (week 8) for FMDV15 specific IgG1 (µg/ml).

Q1 and Q2 represent the lower and upper quartiles, respectively.

***The frequency of each allele.**

DRB3 IgG1	**0901*	**1001*	**1601*	**2707*	*DRB3* IgG2	**1001*	**1601*
weeks					weeks		
2	0.031	n.s.	0.035	<0.001	2	0.013	<0.001
4	n.s.	n.s.	0.004	n.s.	4	n.s.	<0.001
8	0.015	0.031	0.023	0.041	8	0.005	<0.001
10	n.s.	0.038	0.007	n.s.	10	0.012	0.003
AUC	0.005	0.009	0.006	0.049	AUC	0.009	<0.001

Table 3.3 Significant *DRB3* alleles for both FMDV15 peptide specific IgG1 and IgG2 responses across time.

AUC: area under the curve; n.s.: not significant.

p values determined by Wald test. Only those alleles with AUC p<0.05 and that had significant values for more than 1 week are shown.

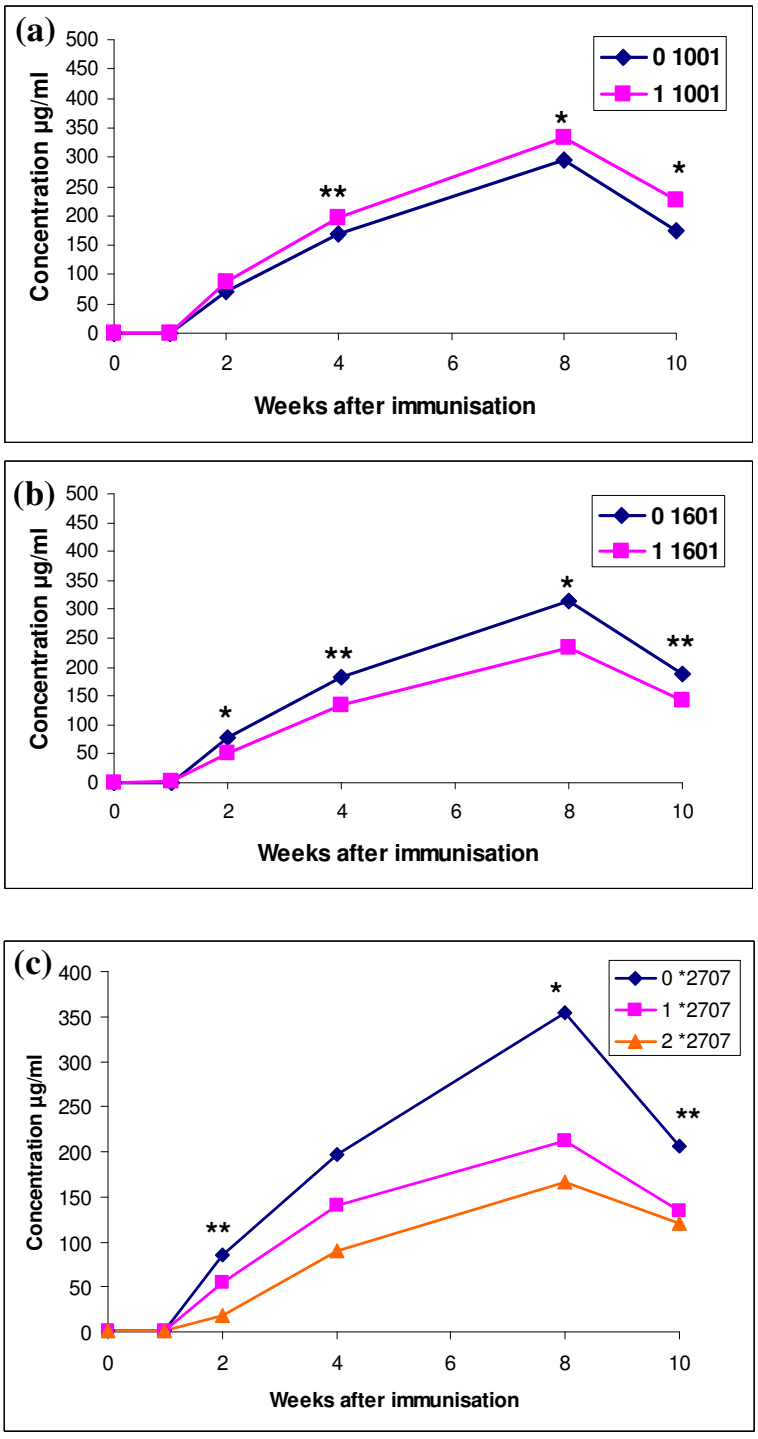


Figure 3.6 (a) anti-FMDV15 IgG1 responses and *DRB3*1001* animals: with 0 copies: ◆ and with 1 copy: ■ (b) anti-FMDV15 IgG1 responses and *DRB3*1601* animals: with 0 copies: ◆ and with 1 copy: ■ (c) anti-FMDV15 IgG1 responses and *DRB3*2707* animals: with 0 copies:◆, with 1 copy: ■ and with 2 copies ▲. p values determined by Student's t-test from predicted means using 0*2707 and 2*2707; **p<0.001, *<0.05.

3.5.5.2 IgG2

IgG2 mean, median, upper and lower quartiles demonstrate that the responses were positively skewed (Table 3.3). On further analysis, it was clear that many of the animals were non-responders at one or more time points. Fifteen of the 178 genotyped animals did not produce any IgG2 response at any of the time points. The majority of these non-responders had alleles *DRB3*2707* (6 of the 15 with 1 homozygote) and *DRB3*1601* (9 of the 15) with 3 animals having both **2707* and **1601*. The mean peak responses were primarily at week 8 although *DRB3*0501, *1001, *2703, *2707* and **3201* had peak values at week 10 (Table 3.3). Animals positive for *DRB3*1001* had the highest mean IgG2 response in the IgG2 data set, and also the highest median value, whereas this allele only ranked 8th in the IgG1 dataset but was still significantly associated with higher IgG1 levels, albeit to a lesser extent. A single copy of this allele was associated with greatly increased mean IgG2 levels at weeks 8 and 10 (Table 3.4 and Figure 3.7a). In contrast, *DRB3 *0901* which was ranked 1st in the IgG1 data set was only ranked 14th in the IgG2 data set, and only the AUC was significant. Animals expressing *DRB3*1601* produced significantly lower levels of FMDV15 peptide-specific IgG2 ($p<0.001$) (Table 3.4) from week 2 onwards (Figure 3.7b) and had a similar ranking for IgG1 and IgG2 (14th and 16th respectively).

DRB3 allele	Frequency*	Number of animals with allele	FMDV15 specific IgG2 Levels (µg/ml)			
			Mean	Median	Lower quartile Q1	Upper quartile Q2
**1001*	0.05	18	80.7#	27.0	17.5	53.6
**1501*	0.02	6	52.3	24.9	12.2	61.3
**0601*	0.06	18	32.7	13.6	2.3	31.4
**0902*	0.04	13	32.2	18.3	0.0	64.3
**1101*	0.16	52	31.5	19.8	8.5	36.3
**1701*	0.01	2	27.0	8.9	0.6	18.4
**1201*	0.06	22	26.9	11.0	0.0	46.6
**0701*	0.02	8	26.5	1.4	0.0	34.3
**0501*	0.04	13	25.6#	10.1	1.2	31.5
**2707*	0.20	68	24.2#	18.8	1.5	34.5
**2703*	0.01	3	23.1#	12.6	4.6	45.4
**3201*	0.01	2	18.7#	12.2	5.6	30.2
**2002*	0.01	4	17.1	9.9	2.1	26.9
**0901*	0.06	22	15.9	4.4	0.0	16.8
**0201*	0.07	25	13.2	0.6	0.0	11.6
**1601*	0.10	37	8.7	7.6	5.4	11.9
**0101*	0.06	21	4.3	4.3	0.0	8.6
**1801*	0.01	4	2.1	2.1	0.0	4.1
Overall	1.0	178	26.0	10.8	0.0	25.8

Table 3.4 *DRB3* alleles in the population and mean, median, upper and lower quartiles at peak week for FMDV15-specific IgG2 (µg/ml).

[#]Week 10 peak values and means. *The frequency of each allele.

Q1 and Q2 represent the lower and upper quartiles, respectively.

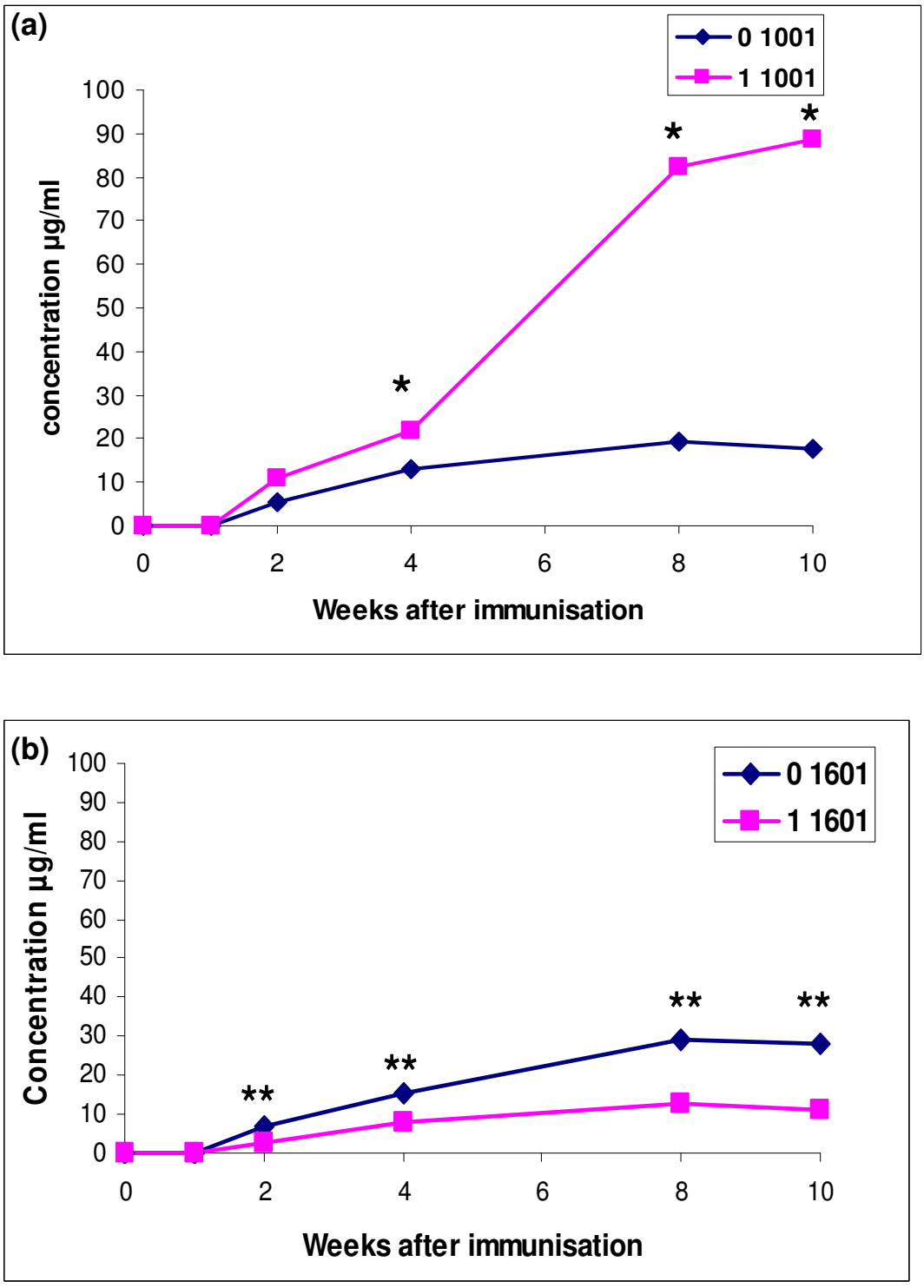


Figure 3.7 (a) anti-FMDV15 IgG2 responses and *DRB3*1001* with 0 copies: ◆ and with 1 copy: ■ (b) anti-FMDV15 IgG2 responses and DRB3*1601 with 0 copies: ◆ and with 1 copy: ■ .
p values determined by Student's t-test from predicted means; **p < 0.001, *< 0.05.

Interestingly, animals positive for the most common allele, *DRB3*2707*, had significantly lower IgG1 and IgG2 responses to the peptide at week 2 ($p<0.001$), although for IgG2, the association was only detected at this week, whereas for IgG1 week 8 was also significant (Table 3.4; Appendix C.2 and C.3).

3.5.6 Association of DRB3 alleles with T cell proliferation to FMDV15 and ConA

3.5.6.1 FMDV15

The T cell mean, median and upper and lower quartiles were examined for the peak week for each of the *DRB3* alleles for response to FMDV15 (Table 3.5). Allele **1001* had the highest T cell SI mean, which is identical to the ranking for IgG2. However **0902* was the lowest mean value for the T cell results and is ranked 4th for IgG2. Both these alleles, *DRB3*1001* and **0902*, were found to be significantly associated with FMDV15 specific proliferation of T cells at both 1.0 µg/ml and 2.0 µg/ml FMDV15 concentrations. For *DRB3*1001*, weeks 8 and 10 were highly significant ($p<0.001$ and $p<0.05$ respectively at both concentrations) (Appendix C.4). Allele **0501* had highly significant ($p<0.001$) results for all FMDV15 concentrations but only at week 0. Animals with **0101* were significant ($p<0.05$) at week 4 for concentrations of 1.0 µg/ml and 2.0 µg/ml. Animals positive for **1101* were significant for week 0 at concentration of 2.0 µg/ml.

	FMDV15 T Cell stimulation index (SI)			
***DRB3* allele**	**mean**	**median**	**lower quartile (Q1)**	**upper quartile (Q2)**
**1001*	18.47	2.42	1.86	9.65
**1801*	15.42	8.46	1.04	29.80
**1501*	9.20	1.85	1.76	14.81
**0701*	9.18	1.38	1.05	6.18
**1601*	7.92	1.31	1.09	2.20
**2707*	4.83	1.52	1.10	2.94
**0101*	4.44	1.82	1.49	5.03
**2703*	4.37#	2.63	2.00	7.18
**0501*	2.69#	1.50	1.08	2.55
**0601*	2.68	1.34	0.94	2.95
**1101*	2.60	1.53	1.21	2.50
**3201*	2.27	2.27	1.19	3.35
**0201*	2.19	1.24	1.07	1.87
**0901*	1.92	1.51	1.06	2.39
**2002*	1.77	1.98	1.01	2.53
**1201*	1.64	1.25	1.08	1.99
**1701*	1.42#	1.42	1.00	1.85
**0902*	1.36#	1.14	0.91	1.27
Overall	2.67	1.44	1.06	2.51

Table 3.5 *DRB3* alleles in the population and mean, median, upper and lower quartiles at peak week for FMDV15 T cell proliferation (SI).

[#] Week 10 peak values and means.

Q1 and Q2 represent the lower and upper quartiles, respectively.

3.5.6.2 ConA

The T cell mean, median and upper and lower quartiles were examined for week 8 so that they were directly comparable to the FMDV peak week for each of the *DRB3* alleles for response to ConA (Table 3.6). Animals positive for allele *DRB3*2002* had the highest mean value whilst animals expressing *DRB3*0902* had the lowest responses. Animals positive for *DRB3*1001* (n=18) had a high response but it was not significant, whilst animals positive for **1701* (n=2) (week 10 $p<0.05$ at 10 µg/ml ConA) had a low response to ConA. Allele *DRB3*1201* had strikingly distinct results, as animals positive for a single copy of this allele did not respond to ConA at any week ($p<0.05$) at both 2.5 µg/ml (results not shown) and 10 µg/ml ConA (Figure 3.8). There were no correlations between the ranking of alleles for response to FMDV and ConA (data not shown).

	ConA T Cell stimulation index (SI)			
***DRB3* allele**	**mean**	**median**	**lower quartile (Q1)**	**upper quartile (Q2)**
**2002*	33.95	37.05	17.29	50.60
**1001*	21.13	8.86	3.82	24.54
**0201*	20.49	13.29	4.51	34.05
**0501*	17.49	5.15	2.28	35.01
**1501*	17.19	15.55	8.03	26.97
**1101*	17.18	8.68	3.60	24.00
**1601*	15.4	10.74	2.63	15.29
**2707*	13.51	6.09	2.96	13.70
**0101*	12.03	7.97	5.97	13.58
**1801*	10.99	9.84	4.07	17.92
**0901*	10.94	5.22	1.58	17.43
**1701*	10.79	10.79	0.55	21.03
**0902*	10.11	3.94	1.63	8.94
**2703*	9.91	4.58	2.04	19.12
**0601*	9.57	3.18	1.37	13.62
**1201*	9.44	4.75	1.70	11.69
**3201*	4.66	4.66	2.32	6.99
**0701*	4.21	3.58	2.41	5.23
Overall	14.08	7.02	2.95	17.94

Table 3.6 *DRB3* alleles in the population and mean, median, upper and lower quartiles at peak week for ConA (10 µg/ml) T cell proliferation (SI).

Q1 and Q2 represent the lower and upper quartiles, respectively.

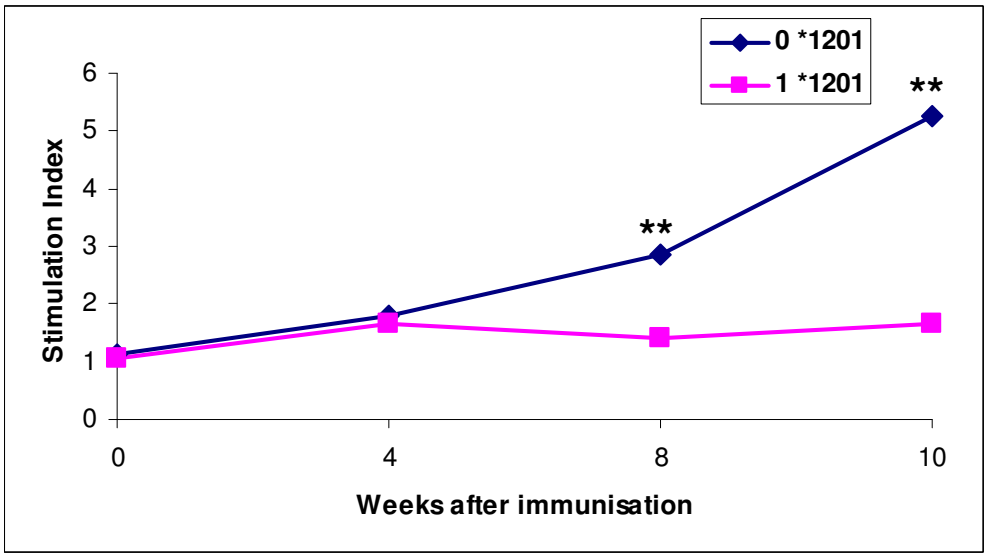


Figure 3.8 T cell proliferation response (SI) to ConA (10µg/ml) and *DRB3*1201* with 0 copies: ◆ and with 1 copy: ■. p values determined by Student's t-test from predicted means; **p<0.001.

3.5.7 Association of *DRB3* alleles with IFN-γ responses to FMDV15 and ConA

3.5.7.1 FMDV15

IFN-γ mean, median, upper and lower quartiles demonstrate that the responses were positively skewed (Table 3.7). The mean peak responses were primarily at week 8 although animals positive for alleles *DRB3*0501*, **1001, *2703, *2707* and **3201* had peak values at week 10 (Table 3.7). On further analysis, it was clear that many of the animals were non-responders at one or more time points. Thirteen of the 178 genotyped animals did not produce any IFN-γ response at any of the time points. The majority of these non-responders were positive for alleles *DRB3*2707* (8 of the 13) and *DRB3*1101* (7 of the 13) with 5 animals being heterozygous for **2707* and **1101*. Only alleles *DRB3*1101* and **1201* were significantly associated with

responses at more than 1 time point. Animals positive for *DRB3*2707* were significantly associated with non-response for week 10 ($p<0.05$). Animals positive for *DRB3*1101* had lower IFN-γ responses ($p <0.001$ at week 4 and $p<0.05$ for week 10), with homozygous animals showing even lower responses than animals which were heterozygous for *DRB3*1101* (Figure 3.9). The predicted means values used in the students t tests were 0**1101* and 1**1101* for week 4 and 0**1101* and 2**1101* for week 10. Animals positive for allele **1201* had $p<0.05$ values at both week 4 and week 10.

	FMDV15 IFN-γ assay			
***DRB3* allele**	**mean**	**median**	**lower quartile**	**upper quartile**
**1801*	2035	417	199	3872
**0101*	1483	399	32	1025
**3201*	1248	1248	108	2388
**1501*	1173	79	0	545
**0501*	953	308	25	459
**0701*	953	679	63	1828
**0601*	881	126	0	361
**2707*	710	219	29	746
**1601*	633	117	0	562
**1101*	627	215	9	440
**0901*	535	369	102	563
**1001#*	502	325	0	854
**1701*	497	497	495	500
**2703*	374	438	196	535
**0201*	268	33	0	323
**0902#*	260	55	0	215
**1201*	253	84	0	429
Overall	679	219	10	600

Table 3.7 *DRB3* alleles in the population and mean, median, upper and lower quartiles at peak week for IFN-γ (pg/ml) response to FMDV15.

[#]Week 10 peak values and means.

Q1 and Q2 represent the lower and upper quartiles, respectively.

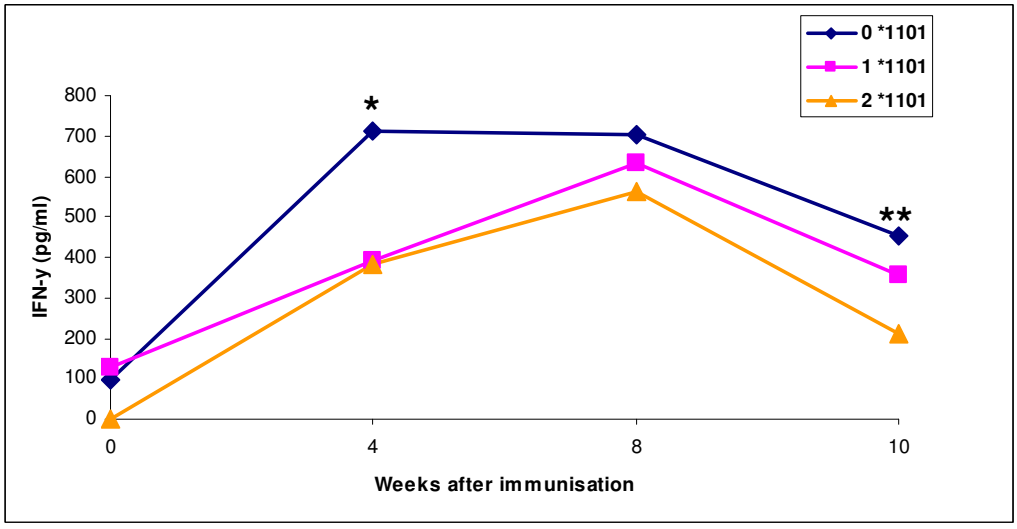


Figure 3.9 IFN-γ response after immunisation with FMDV15 and *DRB31101: 0 copies:◆; 1 copy: ■ and 2 copies:▲. p values determined by Student's t-test from predicted means: **p<0.001 (0**1101* and 2**1101*), *<0.05 (0**1101* and 1**1101*).**

3.5.7.2 ConA

A high number of non-responders were also seen for ConA response, with 12 animals failing to make any detectable IFN-γ at any of time points (5 of the 12 animals also did not produce IFN-γ in response to FMDV either). No alleles were significant for more than one time point, however several alleles were highly significant (p<0.001) at week 4 (**0101, *0501* and **2707*).

	ConA IFN-γ assay			
***DRB3* allele**	**mean**	**median**	**lower quartile**	**upper quartile**
**0501*	1505	666	231	753
**1501*	1490	387	127	788
**0101*	1406	459	238	1022
**1801*	1405	464	220	2590
**3201*	1370	1370	731	2009
**0701*	1306	732	529	2100
**0601*	965	325	38	668
**0902*	861	475	0	685
**1701*	843	843	567	1118
**1601*	821	423	158	819
**2707*	623	354	127	648
**0901*	601	589	141	913
**1101*	569	356	0	688
**1201*	567	446	112	654
**1001*	404	315	0	718
**2703*	398	317	300	517
**0201*	336	101	0	457
Overall	751	405	61	712

Table 3.8 *DRB3* alleles in the population and mean, median, upper and lower quartiles at peak week for IFN-γ (pg/ml) response to ConA.

Q1 and Q2 represent the lower and upper quartiles, respectively.

3.5.8 Summary of trait allele rankings

The correlations for the ranking of the alleles were investigated. None of the allele ranking positions were significantly correlated across any of the traits. However *DRB3*0501* is ranked at 9th place for IgG1, IgG2 and T cell response to FMDV15. In addition alleles **0901*, **1001* and **3201* are all ranked at the same position for IgG2 and T cell response (14th, 1st and 12th respectively).

3.5.9 Non-responders

There was a single animal which failed to make any T cell, IFN-γ or IgG2 response to FMDV15 at any time point. This animal was heterozygous (*0201 and *1101). In addition there was a single animal which did not make a T cell or an IFN-γ response which was homozygous for allele *2707, and two animals which did not make any IFN-γ or IgG2 response which were both heterozygous (*2707, *1601 and *2707, *0101). Thus the *2707 allele may be associated with the non-responders. This allele was also ranked low for IgG1, IgG2 and IFN-γ responses (Table 3.1, 3.2 and 3.7).

3.5.10 Binding pockets

There are several defined peptide binding pockets within the MHC Class II PBC which are encoded by both the alpha and beta DR chains (Bondinas, Moustakas, & Papadopoulos 2007). As the bovine DRB3 molecule has a similar PBC to other species, most of the polymorphisms in the *BoLA DRB3* alleles also encode amino acids found in the pockets (Sharif, Mallard, & Sargeant 2000) (**Chapter 2**).

Pocket 1 position β86 has 3 possible amino acids but most of the animals in the study population had either valine or glycine, with the exception being animals with allele

*DRB3*0101* (n = 20) which has methionine at position β86. Pocket 4 has 5 amino acid positions which are entirely coded by *DRB3*. Pocket 4 position β70 has 3 possible amino acids (glutamic acid, glutamine or arginine) although only a single allele *DRB3*2002* (n = 4), in the study population had a glutamine at this position. Pocket 6 has a wide range of residues possible, with a total of 6 amino acids (serine, histidine, cysteine, threonine, alanine or tyrosine) in the 18 alleles present in the study population. Pocket 7 has 6 positions with position β71 also creating part of pocket 4 and position β61 also forming part of pocket 9. Pocket 9 has 5 positions which show considerable variability in the amino acids present. Pocket 10 forms more of a 'shelf' structure rather than a deep pocket and has several amino acids shared with pocket 9 (Zavala-Ruiz et al. 2004).

The animals were all subdivided according to pockets and results were fitted in the REML model. For IgG1, grouping of animals according to pocket sequence was for the most part not significant, with only pocket 9 position β37 having consistently significant effects (Appendix C.2). However, pocket 4/7 position 71 was highly significant at week 2; individual amino acids were analysed further but were not highly significant (Appendix C.2).

In contrast, for IgG2 the grouping of animals according to the pocket sequence revealed that several positions were associated with significant effects (Appendix C.3). Three of the positions within the pockets of the PBC were associated with the magnitude of the IgG2 levels over the majority of the time points: position β70 (pocket 4), position β57 (pocket 9) and position β61 (pocket 7 and 9) (Table 3.9).

Pocket	Amino acid	No. of animals	Mean	Median	Lower quartile	Upper quartile
4 position 70	E	119	23.9	9.0	0.0	20.9
	R	137	30.5	12.3	1.5	31.4
	Q	4	32.2	18.3	0.0	64.3
9 position 57	D	161	25.5	10.0	0.0	25.1
	V	48	50.6	2.2	10.6	50.5
	A	4	8.7	7.6	5.4	11.9
	S	52	25.2	9.9	1.4	31.4
7/9 position 61	C	17	86.7	30.5	17.5	67.0
	W	172	26.5	11.3	0.0	26.0
	L	23	22.4	11.6	0.3	25.9

Table 3.9 IgG2 (µg/ml) significant binding pockets/amino acids present with the mean, median and lower and upper quartiles at week 8.

In terms of the T cell proliferation to FMDV15, there were a few pocket positions which were highly significant ($p<0.05$). Within pocket 7, positions 61, 71 and 76 and within pocket 9, positions 37, 57 and 61 were all significant. However only 1 position was significant for the T cell proliferation to ConA which was position 28 within pocket 7 (Appendix C.4 and C.5)

In contrast to this, there were many positions within the PBC which were highly significant for the IFN-γ response (Table 3.10).

Pocket	Position	Week	p values (FMDV)	Week	p values (ConA)
1	86	4	0.013	10	<0.001
4	13	n/s		8	0.001
	70	4	0.005	n/s	
		n/s		8	<0.001
		10	0.045	n/s	
4/7	71	4	<0.001	n/s	
		10	0.005		
4	74	4	<0.001	n/s	
		10	<0.001		
6	11	8	0.027	8	<0.001
7	28	8	0.018	n/s	
		10	<0.001		
	30	10	<0.001	8	<0.001
	47	4	<0.001	n/s	
7/9	61	10	<0.001	10	<0.001
7	67	10	<0.001	n/s	
9	9	4	0.008	8	0.007
				10	<0.001
	37	4	0.031		
		8	<0.001	8	0.004
	57	4	0.005	4	<0.001
		8	0.031	8	0.008
		10	<0.001	10	<0.001
	60	10	<0.001	10	<0.001
10	56	4	<0.001	n/s	
		8	<0.001		
		10	0.027		

Table 3.10 Significant DRB3 pocket positions and p values for IFN-γ levels in response to FMDV15 and ConA.

3.5.10.1 DR molecule: binding pocket 1

The IFN-γ response at week 4 for the FMDV15 peptide indicated that pocket 1, position 86, had a significant effect (p= 0.013) (Table 3.10). For the ConA analysis, pocket 1 was also highly significant (p< 0.001), but at a later time point (week 10). At this position there can be an M, V or G (glycine). However, as only 1 animal has the M/M genotype, the results should be treated with caution.

3.5.10.2 DR molecule: binding pocket 4

The effects of pocket 4, which is formed from amino acids at positions β13, 70, 71, 74 and 78, were analysed using the REML model (Appendix C.2 and C.3). Position β70 was significantly associated with FMDV-specific IgG1 (p<0.001 at week 2) and IgG2 (p<0.001 at weeks 2, 4 and with AUC) (Appendix C.2 and C.3). Amino acids present at β70 are either a glutamic acid (E) (n= 119), an arginine (R) (n= 137) or rarely in this data set glutamine (Q) (n= 4). Thus, the IgG1 response both before and after the boost was strongly correlated with the presence of either glutamic acid or arginine at position β70. Animals homozygous for E at position β70 produced significantly lower levels of IgG1 and IgG2 over most time points compared to animals homozygous for R at this position (p<0.001) (Figure 3.10a). Furthermore, there was no evidence of boosting of the IgG2 response in animals homozygous for E, which by week 8 were only producing about one quarter of the IgG2 levels compared to animals homozygous for R (p<0.001) (Figure 3.10b). The IFN-γ response to the FMDV15 peptide was also significant at β70. In contrast to the antibody response, animals with the R/R genotype had lower levels of IFN-γ

compared to animals which were heterozygous (E/R) (Figure 3.10c). The T cell proliferation was also significant for both E and R at week 4 ($p<0.05$). The predicted means values used in the Students t-test was between the two homozygotes (two copies of arginine and two copies of glutamic acid at position β70) for all figures.

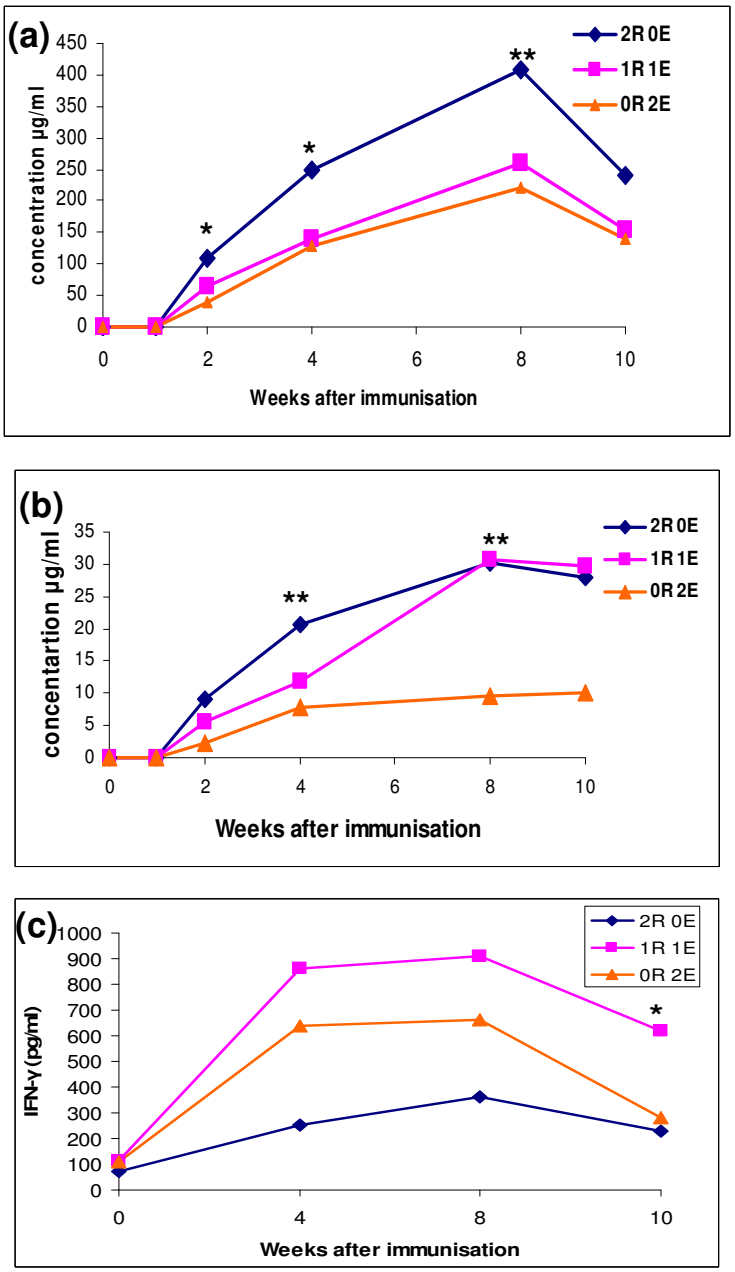


Figure 3.10 Pocket 4 position β70. (a) anti-FMDV15 IgG1 responses and position β70 homozygous for glutamic acid (E) ▲, heterozygous for E and R □, or homozygous for arginine (R) ♦. (b) anti-FMDV15 IgG2 responses and position β70 homozygous for glutamic acid (E) ▲, heterozygous for E and R □, or homozygous for arginine (R) ♦. (c) IFN-γ levels and position β70 homozygous for glutamic acid (E) ▲, heterozygous for E and R ■, or homozygous for arginine (R) ♦. p values determined by Student's t-test from predicted means: **p<0.001, *<0.05. The predicted means used for the Student's t-test was between the two homozygotes.

3.5.10.3 DR molecule: binding Pocket 7

All positions in pocket 7 had at least 1 week which was highly significant ($p<0.001$) for IFN-γ levels. However, most of the pocket 7 positions have many amino acid combinations. For position β67 there are 5 combinations of amino acids, homozygous F/F or I/I or heterozygous F/I, F/T or I/T. Being positive for F/T at β67 resulted in higher IFN-γ levels than animals positive for I/I across all time points Figure 3.11. The animals positive for I/I had the lowest mean IFN-γ levels compared to all other amino acid combinations at this position. The Students t-test analysis on the predicted means showed that the mean of animals with the F/F genotype was significantly different to the means of the animals with I/T at week 8 ($p<0.001$). The

The T cell proliferation had several positions within pocket 7 which were significant; β28, 61, 67 and 71 ($p<0.05$) at weeks 8 and 10 (Appendix C.4 and C.5).

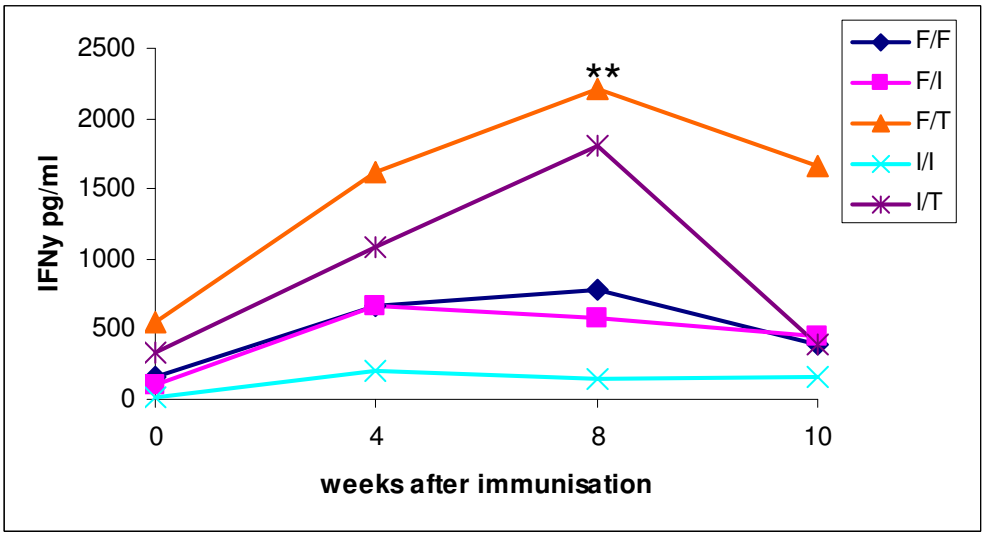


Figure 3.11 Pocket 7 position β67 and IFN-γ response to FMDV15 (pg/ml): Homozygous F/F ◆, heterozygous F/I ■, heterozygous F/T ▲, homozygous I/I × and heterozygous I/T ✳ . p values determined by Student's t-test on F/T and I/I from predicted means: **p<0.001, *<0.05. F/F and I/T predicted means values used in students t-test.

3.5.10.4 DR molecule: binding Pocket 9

Pocket 9 was significantly associated with IgG1 and IgG2 levels (Table 3.9). Pocket 9 consists of β9, 37, 57, 60 and 61. A significant effect was found at all of these positions, except β9, for IgG2 (Appendix C.3). The significant positions were analysed further for both IgG1 and IgG2. Position β37 amino acids include tyrosine (Y) (n=81), phenylalanine (F) (n=99), threonine (T) (n=84), arginine (R) (n=5), and asparagine (N) (n=34). N was significant for IgG1 (p=0.047), but for IgG2, T and N were both significant (p=0.006 and p=0.032 respectively). Having a T at position β37 was associated with lower levels of IgG2, whilst having an N was associated with higher levels of IgG2 (Appendix C.3).

At position β57, the amino acid possibilities were alanine (A) (n=4), aspartic acid (D) (n=161), serine (S) (n=52) or valine (V) (n=48) (Table 3.9). The mean FMDV15 specific IgG2 values for V/V, D/D and V/D were 23.91, 13.42 and 52.64 μg/ml respectively (Figure 3.12). The Student's t-test to calculate the significance levels was between the D/D and V/D predicted means. Strikingly, the animals which were heterozygous for the V/D genotype produced higher levels of FMDV15 specific IgG2 whereas all other combinations resulted in lower levels ($p<0.05$ at weeks 4 and 10, and $p<0.001$ at weeks 8 and 10). The highest mean value for the 48 animals possessing a V at position 57 (50.6 g/ml in Table 3.9) in fact reflected the disproportionate contribution by V/D heterozygous animals in this grouping.

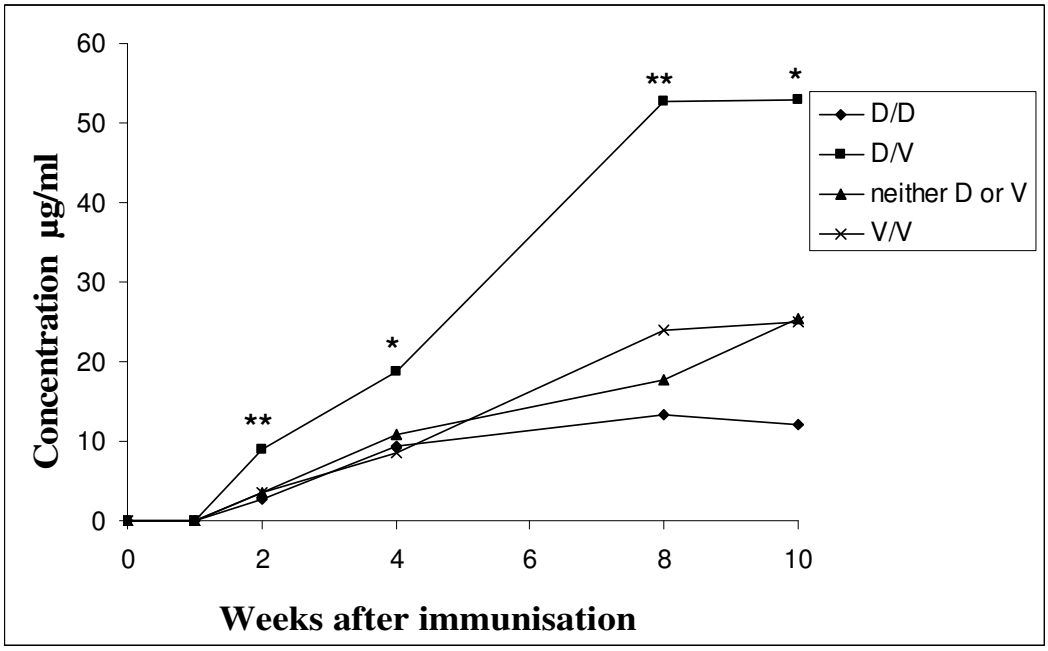


Figure 3.12 Pocket 9 position β57 and anti-FMDV IgG2 responses: neither D nor V▲, homozygous for V X, V/D ■ or homozygous for D ♦. p values determined by Student's t-test from predicted means:p<0.001, *<0.05. D/D and D/V predicted means values used for the Student's t-test.**

At position β61 there can either be a cysteine (C) (n= 17), leucine (L) (n= 23) or tryptophan (W) (n=172) (Table 3.9). Both C and W were associated with both IgG1 and IgG2 levels ($p<0.05$; data not shown). Further analysis revealed a complex relationship between alleles as animals heterozygous for W produced significantly higher levels of IgG2 than any other combination yet animals homozygous for tryptophan had the lowest response (Figure 3.13). The 0W and 2W predicted means values were used in the Student's t-test analysis. In particular, animals with the genotype C/W had the highest response of all and this effect was particularly pronounced during the secondary response (mean C/W week 10, 97.3g/ml).

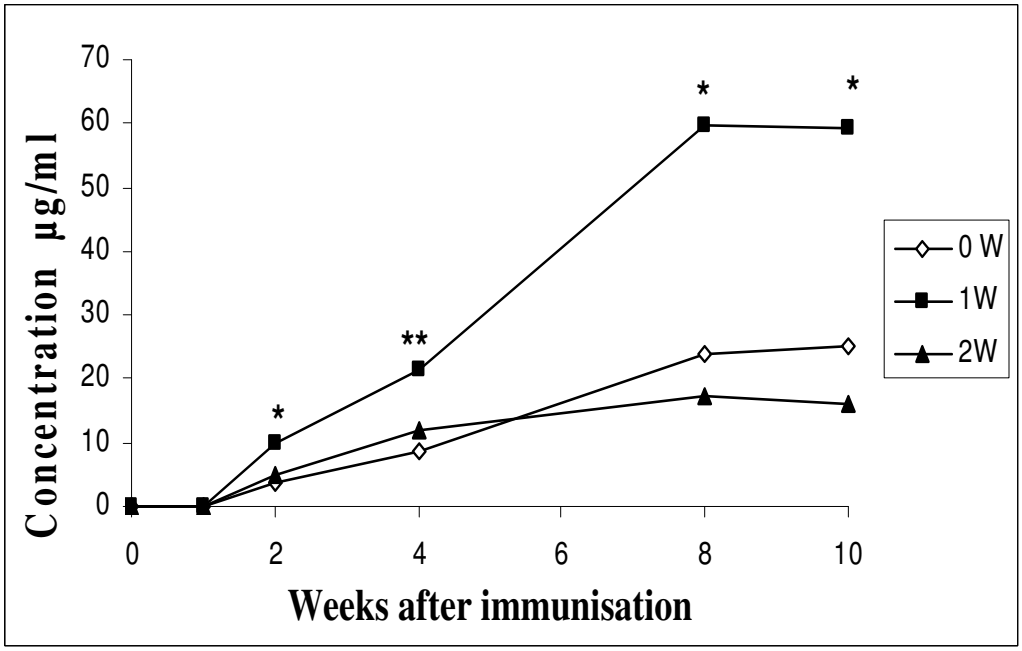


Figure 3.13 Pocket 9 position β61 and anti-FMDV IgG2 responses: no copies of W ◊, single copy of W ■ or homozygous for W ▲. p values determined by Student's t-test from predicted means: **p<0.001, *<0.05. 0W and 2W predicted means used in the Student's t-test

3.5.10.5 DR molecule: binding Pocket 10

Pocket 10, which consists of β56, 57 and 60, overlaps with pocket 9. Position β56 is significantly associated with IgG2 levels but not IgG1 (Appendix C.2 and C.3). Amino acids possible at this position include proline (P), R or Q. Both Q and R were significantly associated with IgG1 and IgG2 response, with a single copy of the R having a highly significant effect for specific IgG2 (data not shown). Having an R was associated with an increase in IgG2. The IFN-γ response was highly significantly associated with β56 with weeks 4, 8 ($p<0.001$) and week 10 ($p<0.005$) and an R at this position is highly significant ($p<0.05$).

The remaining pockets were also analysed (Appendix C.2, C.3, C.4 and C.5). Pocket 1 (position β86) had significant effects on IgG1 antibody levels ($p<0.05$) at week 10 and IgG2 weeks 4 and 8. Pocket 4 position β13 was significant at weeks 4 and 8 for IgG2, but at no time points for IgG1. Position β74 was only significant for a single time point, week 8 for IgG1 and week 2 for IgG2. Pocket 6 β11 was significant at week 2 for IgG1 ($p<0.05$) and IgG2 ($p<0.001$). None of the positions within pocket 7 were significant for IgG1. However, for IgG2 there are several pocket 7 positions significant for a single time point (β28, 30, and 71). The rest of the pocket positions were not significant (Appendix C.2, C.3, C.4 and C.5).

3.5.11 3D modelling

Overall it seemed apparent that the pocket positions had a profound influence on the ensuing immune response, and so it would seem highly probable that this is due to the binding affinity. As there is no crystalline structure for the bovine DR molecule,

modelling was attempted using the HLA-DR molecule as a template. The modelling of both the BoLA-DR molecule and the peptide represents very preliminary data.

The alleles **1001* and **1601* were highly significantly associated with the antibody response to FMDV15, with **1001* being positively associated and **1601* negatively associated. The preliminary data from the modelling of these two alleles show that the structure of the PBC is different in the two alleles. The two alleles differ most in pocket 4, where the DRB3*1001 has an overall positive electrostatic charge (Figure 3.14) whereas *1601 has an overall negative electrostatic charge (Figure 3.15). These electrostatic differences were most likely determined by the negatively charged glutamic acid and positively charged arginine (DRB3*1601 and DRB3*1001 respectively) at position β70, within pocket 4. These were significantly associated with the IgG1, IgG2 and IFN-γ response to the FMDV15 peptide. The negatively charged glutamic acid at β70 may possibly be altering the binding of the FMDV15 peptide resulting in different levels of response. Due to difficulties in correctly modelling a small 40-mer peptide, it is not possible to accurately predict the docking of the peptide with each of the two alleles, although very preliminary data (not shown) indicates that this may be the case.

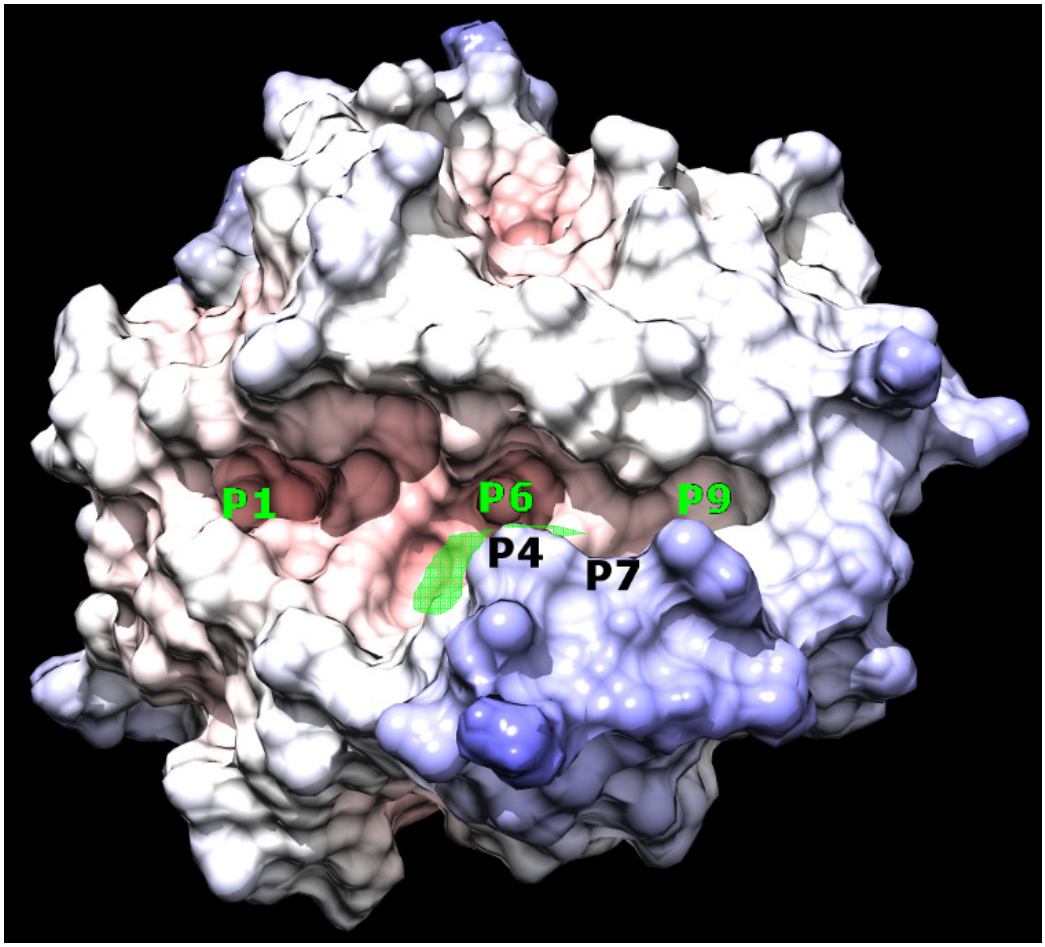


Figure 3.14 3D electrostatic model of DR molecule using *DRB3*1001.* Blue represents an overall negative charge, red represents an overall positive charge and white represents an overall neutral charge. Pockets 1, 4, 6, 7 and 9 are shown within the PBC. Pocket 4 is highlights in the green shading.

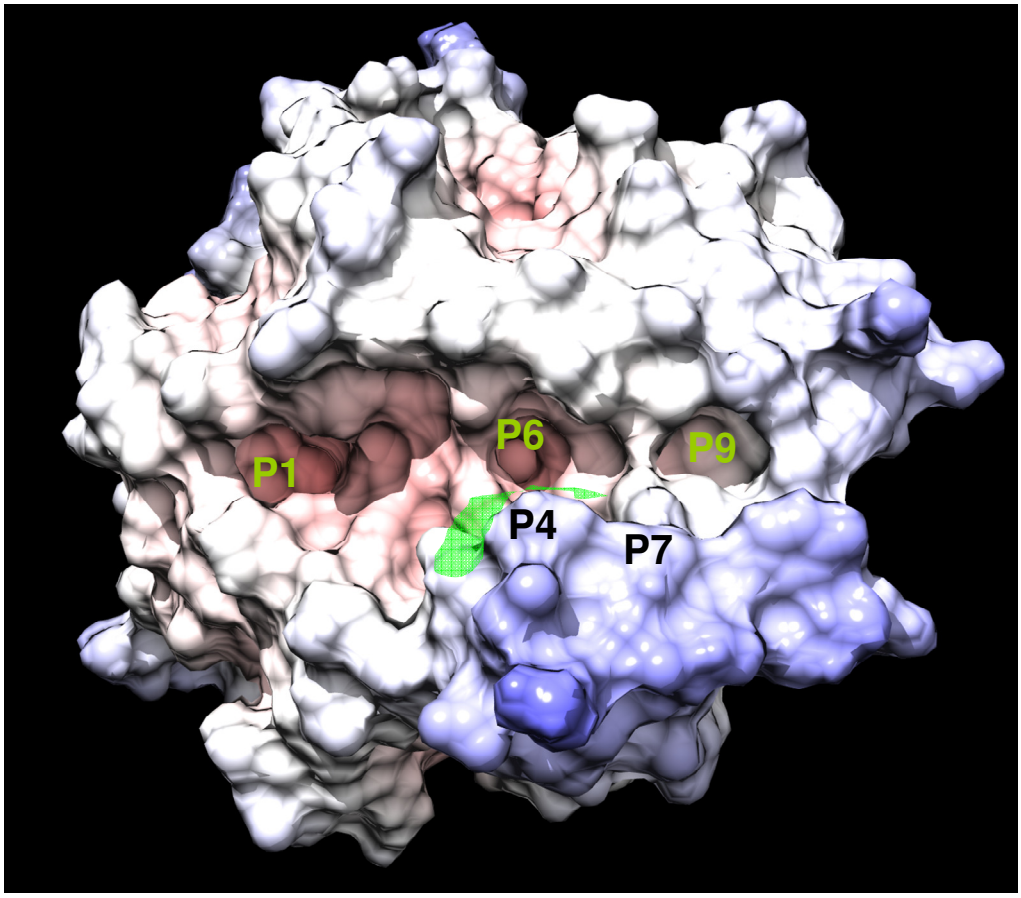


Figure 3.15 3D electrostatic model of DR molecule using *DRB3*1601.* Blue represents an overall negative charge, red represents an overall positive charge and white represents an overall neutral charge. Pockets 1, 4, 6, 7 and 9 are shown within the PBC. Pocket 4 is highlights in the green shading.

3.6 Discussion

For a vaccine to be considered successful an appropriate protective immune response has to be elicited in all individuals. Results in this chapter demonstrate that several *BoLA-DRB3* alleles are significantly associated with the level of the adaptive immune response produced after immunisation with a synthetic peptide of the FMDV VP1 capsid protein. Furthermore, individual positions within the PBC are significantly associated with the levels of the specific antibody and IFN-γ, and to a lesser extent T cell, response to FMDV15.

Even though the immunising antigen, FDMV15, is relatively simple compared to a pathogen or subunit vaccine, a considerable degree of variation is seen in the responses to the FMDV15 peptide post-immunisation, suggesting that not all animals are protected to the same degree. Previous studies have indicated that *DRB3* alleles are associated with variation in response to FMDV (Glass et al. 1991; Glass & Millar 1994; Garcia-Briones et al. 2000) and with protection against viral challenge following immunisation with FMDV peptides (Glass et al. 1991; Garcia-Briones et al. 2000).

BoLA-DRB3 is one of the most polymorphic genes within the bovine *MHC* locus and *BoLA-DRB3* polymorphisms were more strongly linked to variation in the IgG2 response and IFN-γ response than to the IgG1 response, with the fewest associations found for the T cell proliferation responses. This might suggest that genes other than *BoLA-DRB3* have greater impact on the variation in the IgG1 and T cell responses. However, it should be noted that the assays for the T cell and IFN-γ responses were

conducted with whole blood, and proliferation for logistical reasons was measured after 6 days of culture which is not optimal. Furthermore it is also possible that at the later time points rising antibody levels in the blood may have inhibited the T cell and IFN-γ response by binding to the peptide antigen. Nevertheless, significant associations were found for the cell-based assays. The bovine antibody isotype functions are not as fully described as human or mouse but it is thought that the IgG1 contributes to phagocytosis whilst an IgG2 response has a neutralising effect. The switch between the IgG subclasses is complex and is not entirely understood, but it is hypothesised the affinity of peptide/MHC binding dictates the resultant IgG response (Mulcahy et al. 1990; Estes & Brown 2002). It is possible that the binding affinity of processed peptides may be different between different *DRB3* alleles which might result in either an IgG1-biased or an IgG2-biased response. It is interesting to note that the IgG2 and IFN-γ responses (which are Th1) have a more significant association with the alleles of *DRB3*.

There was evidence that certain alleles and amino acids in pockets 4 and 7 had different relationships with the primary and secondary responses, suggesting that *DRB3* polymorphisms may also influence the duration of response. The level of IFN-γ is correlated with the ability of a vaccinated animal to control replication of the virus (Parida et al. 2006), and there are many positions within the PBC which are highly significantly associated with the levels of IFN-γ post-immunisation.

A number of animals responded to the FMDV15 peptide at week 0 for T cell proliferation and IFN-γ production, and this was unexpected as the animals were naïve to FMDV. A possible explanation for this may be the binding of the peptide to integrin receptors on the cell surface. FMDV is known to interact with these

receptors through the RGD loop on the viral capsid (Berinstein et al. 1995) which is also present in the FMDV15 peptide. It may be that this interaction between the peptide and the integrin receptors triggered the low level T cell proliferation and IFN-γ production in week 0.

Although many of the associations described in this chapter are highly significant, they are not absolute, which would indicate that there are other factors playing a role in determining the variation in immune responses to the FMDV15 peptide. As the BoLA region, like other vertebrate species, is extremely gene dense with at least 154 genes expressed (The Bovine Genome Sequencing and Analysis Consortium et al. 2009), it is possible that the associations with *BoLA-DRB3* polymorphisms are, at least partly, due to the influence of other linked loci, in particular *DQA* and/or *DQB*, both of which are polymorphic in cattle (Lewin, Russell, & Glass 1999). In fact, it has been shown that peptides derived from FMDV can be presented by DQ molecules (Glass, Oliver, & Russell 2000; Gerner et al. 2009). Certainly *DR-DQ* linked haplotypes have been described (http://www.ebi.ac.uk/ipd/mhc/bola; Lewin, Russell, & Glass 1999), but the majority of these haplotypes have been described for single breeds, and do not have a one to one relationship, as there are many more *DRB3* alleles than *DQA* or *DQB* alleles. For example within the Holstein breed, *DQA*0101/DQB*0101* was present on a haplotype containing *DRB3*0101* as well as a haplotype containing *DRB3*2002* (Glass, Oliver, & Russell 2000). In this chapter there were two *DRB3* alleles (**0101* and **2002*) were associated with different responses to the FMDV peptide, suggesting that the DQ restricted presentation could not account for the differential responses. In addition, as the population was a cross derived from a breed selected for beef qualities (Charolais)

and a breed selected for dairy characteristics (Holstein), it may be inappropriate to infer the *DR-DQ* haplotypes as these may also differ between the breeds. However the contribution of other loci in this study cannot be ruled out. The observed variation across the population is also partly accounted for by host genetics at other non-MHC loci (and partly by environmental factors) (Leach et al. 2010). In addition, DRB3 molecules can present FMDV-derived epitopes directly to bovine T cells using DR transfectants (Fraser et al. 1996) and anti-DR blocking mAbs (Fraser et al. 1996; Glass, Oliver, & Russell 2000). The FMDV15 peptide used here is unlikely to bind to BoLA Class II molecules directly as it is a 40-mer, but is likely to be processed into subpeptides with the 140–160 bp region being immunodominant at least for T cell responses (Glass et al. 1991; Glass & Millar 1995). Previous studies have indicated that *DRB3* alleles are associated with variation in T cell response to FMDV peptides (Glass & Millar 1994; Garcia-Briones et al. 2000) and others have shown that the binding affinity of some FMDV peptides to certain *BoLA-DRB3* alleles is correlated with T cell proliferation (Haghparast et al. 2000). T cell responses also correlate with serum neutralising titre (Glass & Millar 1994; Glass, unpublished observations) and with protection against viral challenge following immunisation with FMDV peptides (Garcia-Briones et al. 2000). Nonetheless epitopes derived from FMDV15 peptide may be different from those derived from the native virion (Van Lierop et al. 1995) and FMDV15-specific T cells primed in *DRB3*1801* positive cattle were directed to the PPS spacer region (Glass & Millar 1995), which may explain why *DRB3*1801* positive animals were not protected by similar peptides (Garcia-Briones et al. 2000). Data presented in this chapter, although based on only four animals, would tend to confirm that *DRB3*1801* is associated

with non-protection as it ranked as the lowest for both FMDV15 peptide-specific IgG1 and IgG2, and yet is ranked 2nd for T cell response. Unfortunately logistics did not allow a fine mapping of T cell epitopes in this study, but it would have been interesting to determine if the animals positive for *DRB3*1801* were indeed focused on the spacer region. It would further support the premise that the observed associations are DR-specific as similar results were also seen in both Argentinean Hereford cattle (Garcia-Briones et al. 2000) and UK Holstein–Charolais cross breeds (this study). The most frequent allele in the RoBoGen population is **2707*. However, this allele seems to be associated with a lack of response of IgG2 and IFN-γ.

Although a humoral response is generally accepted as the main protective mechanism against FMDV (Goris et al. 2008), the correlation between the levels of neutralising antibody and protection are not always apparent, especially in the case of peptide or subunit vaccines, and it is clear that other factors play a role (Collen, Dimarchi, & Doel 1991). Indeed it is clear that innate, cell mediated and different aspects of humoral immunity all play a role in determining whether an animal is protected against FMDV (Collen 1994; Sobrino et al. 2001; Summerfield et al. 2009). Furthermore, it is likely that a combination of Th1 and Th2 responses are necessary to provide protection as both IgG1 (Capozzo et al. 1997) and IFN-γ (Parida et al. 2006) (which in cattle are associated with Th2 and Th1 responses respectively (Estes & Brown 2002)), correlate with protection. However in none of these studies has the role of host genetics been considered, even though considerable animal to animal variation was observed. By using a large number of animals with a consistent immunisation regime it has been shown here that *BoLA-DRB3* polymorphisms are linked to both cell-mediated and humoral responses to FMDV15.

Further research in this area is clearly warranted as neither the antiviral antibody or protection against viral challenge has been measured, and different alleles were associated with each type of response.

The relative balance of humoral and cell-mediated immunity as well as the isotypes of IgG, IgG1/IgG2, may dictate whether protective immunity is generated, as the ratio between the type of isotypes produced may be dependent on the binding affinity of the peptide to the MHC molecules. It would be feasible to test this hypothesis, for example using animals with allele *DRB3*0901* and investigating any differences in IgG1/IgG2 ratios and protection levels after immunisation, as animals with this allele showed high mean levels for IgG1 but lower levels for IgG2 compared to the rest of the herd. The **1001* allele would be of use for further investigations, as this allele is top of the ranking for both IgG2 and T cell responses. It was also a good candidate for protection against viral challenge following FMDV-derived peptide immunisation (Garcia-Briones et al. 2000). These findings suggest that the DRB3 alleles would be an important consideration in the design of any synthetic vaccine.

There is likely to be a correlation between peptide binding affinity to restriction elements and the resultant immune response and indeed another study has shown that binding affinity of FMDV-derived peptides is correlated with T cell proliferation (Haghparast et al. 2000). Polymorphisms present within *DRB3* exon 2 result in numerous amino acid changes in the PBC. Specific positions within the PBC form 'pockets' where side chains of the FMDV15 peptide bind. The ability of a pocket to 'anchor' a peptide is due to the electrostatic charges of the pocket region and the electrostatic charges of the peptide (Stern et al. 1994). Several of these pocket positions were significantly associated with FMDV-specific IgG1 and IgG2 levels.

Binding pocket 4 in particular has a significant impact on the IgG antibody level, and on the IFN-γ levels, which is likely to be due to its central position within the PBC (Fu et al. 1995; Cardenas et al. 2005). Pocket 4 is entirely encoded by exon 2, and includes five amino acids which together create a wide variety of amino acid motif possibilities. However, a single position, β70, had the most significant association with IgG1 and IgG2 response and was also significantly associated with the cellular responses. The change at β70 from a negatively charged glutamic acid to a positively charged arginine in allele **1001* and **1601* alters the conformation of the PBC in the 3D models (Figure 3.14 and 3.15) and potentially impacts on the binding of the peptide within the PBC. This may explain the strong association with the IgG and IFN-γ responses. E and R at β70 were significantly associated with the T cell proliferation at an early time point of week 4 perhaps suggesting that other factors are affecting the secondary T cell response. These results suggest that the relationship between *BoLA-DRB3* polymorphisms and immune response may be because of differences in the strength of binding of peptide to the PBC in different alleles. More detailed molecular studies are required to ascertain if this is the case.

The amino acids present in pocket 4 also influence the immune response to other pathogens. Positions β13, β71 and β74 are associated with mastitis resistance (Sharif, Mallard, & Sargeant 2000), and the amino acid motif ER at β70 and β71 was associated with resistance to persistent lymphocytosis (Xu et al. 1993). Resistance to dermatophiliosis in Zebu cattle has been associated with the EIAY motif at positions β66-β67-β74-β78, which includes pocket 4 (Maillard, Martinez, & Bensaid 1996). The deletion of β65 in *DRB3,* present in allele *DRB3*0201,* affects the conformation of pocket 4 and the resultant immune response to a vaccine against ticks (Sitte et al.

2002). In humans significant associations have been found between the *DRB* allele and resulting immune responses, e.g. HLA-DR pocket 4 has been linked to resistance to tuberculoid leprosy (Zerva et al. 1996). A recent study on the diversity within the PBC in *DRB3* alleles has revealed that pocket 4 and particularly position 70 in cattle are under greater positive selection than other mammalian species (Takeshima et al. 2009), underlining the potential importance of pocket 4 for vaccine design.

In a previous study, a trend towards protection against viral challenge following FMDV peptide immunisation was observed with alleles *DRB3*1, *3* and **7* (**0501, *1001* and **0201* respectively) (Garcia-Briones et al. 2000). These alleles all have arginine at β70, which together with the data presented here provides further evidence that this residue may be critical for protection against FMDV challenge. Therefore potentially a more effective vaccine could be designed that comprised two separate FMDV peptides that bind to both types of amino acid at position β70. Such a vaccine may result in FMDV protection in the major part of the population described here. However, at β70 there are two further possible amino acids: aspartic acid (negatively charged) and glutamine (neutrally charged) (http://www.ebi.ac.uk/ipd/mhc/bola/). Nonetheless, it may be feasible to create a vaccine containing different versions of the epitopes which would bind to a positive, negative or neutral charge at β70 and thus potentially elicit a universal response in all cattle populations. This could be formally tested by immunising and challenging appropriately typed cattle.

Positions in pockets 7, 9 and 10 were also found to be significantly associated with the antibody response. Two of these, β37 and β57 in pocket 9, were also associated with the IgG1 responses, and both of these residues are also under positive selection in cattle (Takeshima et al. 2009).

All the pockets in the PBC were found to have a profound impact on the IFN-γ levels for both the response to ConA and the FMDV15 peptide. The IFN-γ levels were more significant than any of the other measured immune responses. There is no available literature examining the association between IFN-γ response and MHC alleles.

Pocket 10 is not usually considered when investigating the PBC, however in human DRB the polymorphisms present at positions β56, 57, 76 and 60 (pocket 10) were found to be important for peptide binding (Zavala-Ruiz et al. 2004). In the present study position β56 in pocket 10 was associated with IgG2 and IFN-γ levels, and the positively charged arginine at this position had the greatest effect on the levels of antibody and IFN-γ. Therefore several of the binding pockets, other than pocket 4, have the potential to significantly affect peptide binding affinity, and it would also be prudent to consider other T cell epitopes and their interactions with DR and DQ molecules in the design of any new vaccine to maximise the population response. Further studies are now warranted to test our predictions that DRB3 type is related to the level of response. As pocket 4 seems statistically to be the most important it may represent the major host-derived consideration for future peptide vaccine designs.

MHC heterozygotes are hypothesised as having a selective immunological advantage over homozygotes, although the evidence for this has been mixed (Penn, Damjanovich, & Potts 2002). MHC heterozygote advantage has been described in human diseases such as those caused by Hepatitis B and HIV infection (Thursz et al. 1997; Carrington et al. 1999). This benefit may be due to the increased diversity of peptides being presented to T cells and thus generating a more diverse T cell population (Penn, Damjanovich, & Potts 2002). In the cattle population used in this study most of the animals were heterozygous at the *DRB3* locus (~89%), therefore it is statistically difficult to investigate heterozygote advantage. However, by investigating the individual pocket positions within the PBC, the level of complexity is decreased, which enabled the population to be divided into larger subsets than those for the individual alleles. Using this approach significant heterozygote advantage at two positions was identified: animals heterozygous at β57 (within pocket 9 and 10) for valine and aspartic acid had a higher IgG2 response compared to either of the homozygotes (Figure 3.10) and in addition, animals heterozygous for tryptophan/cysteine at position β61 also had a greater IgG2 response compared to homozygous animals (Figure 3.11). A heterozygote advantage was also observed for the levels of IFN-γ, within pocket 7 (Figure 3.9). These examples were the only evidence for heterozygote advantage seen in this population. However it supports the premise that maintaining diversity in MHC alleles is important for greater disease resistance, although the limited use of particular sires in modern cattle herds may preclude such benefits.

In this chapter further evidence is presented that the limited success of the FMDV15 peptide in inducing protection against the virus may be due to the polymorphisms present in the *BoLA-DRB3* locus. It has been demonstrated that the *BoLA-DRB3* gene affects the response to FMDV15 even in a diverse genetic background. Although it has not been shown whether the alleles directly affect the level of protection, it has been shown that the magnitude and nature of peptide-specific antibody was influenced by the polymorphisms present at the *DRB3* locus. In addition it was demonstrated that the polymorphisms in the binding pockets, in particular pocket 4, mainly the β70 position, where there is a change from negative to positive charge, are likely to have the greatest impact in significantly altering the IgG and IFN-γ responses. However, the role that the binding pockets play in the T cell proliferation is likely to be more complex and other factors may be involved. Thus it may be possible to design an effective FMDV peptide vaccine, based on the conformation of the PBC, and the charges of the key amino acids in the population of cattle, such that that the majority of animals would be protected. Similar considerations could be adapted and used for the design of other vaccines.

4 *BoLA-DRB3* alleles and polymorphisms and associations with the IgG to respiratory vaccines and association with peripheral blood mononuclear cell proliferation to *Staphylococcus aureus* and phytohaemagglutinin (PHA)

4.1 Introduction

The results from **Chapter 3** indicated that alleles of *DRB3* and polymorphisms in the PBC had significant associations with the response to immunisation with a synthetic 40-mer peptide derived from FMDV (Baxter et al. 2009). A number of studies have also found evidence for diversity in *DRB3* impacting on response to pathogens and other antigens (Xu et al. 1993; Garcia-Briones et al. 2000; Udina et al. 2003; Ballingall et al. 2004). It is thus reasonable to hypothesise that *DRB3* alleles and PBC polymorphisms may also have significant associations with responses to commercially available vaccines and commonly occurring pathogens and that the experimental design described in **Chapter 3** would facilitate such a study. Associations between *DRB3* alleles and the PBC polymorphisms and response to two respiratory vaccines for bovine respiratory syncytial virus (BRSV), parainfluenza virus-3 (PIV3) and bovine herpes virus-1 (BHV1) (also known as infectious bovine rhinotracheitis virus (IBRV)) were explored in this chapter. In addition *DRB3* allele associations with the response to a *Staphylococcus aureus* strain and to a mitogen, phytohaemagglutinin (PHA), within the same cattle population were studied.

4.1.1 Bovine respiratory disease

Bovine respiratory disease (BRD) has a major economic and animal welfare impact worldwide and is estimated to cost the UK livestock industry more than £60 million per year (NADIS 2010). BRD is a complex of diseases with many viral and bacterial infectious agents. The viral pathogens which are known to contribute to the development of BRD include BRSV, PIV3, BHV1 and bovine viral diarrhoea virus BVDV. Often infection with viral respiratory pathogens results in secondary bacterial infections. Currently the control methods for BRD include vaccination against the four viral pathogens (Bowland & Shewen 2000), as well as vaccination against the bacterial pathogens *Mannhiemia haemolytica* and *Pasteurella multocida,* associated with BRD (Fulton et al. 2004), although the efficacy of these vaccines has mainly not been subject to controlled challenge studies (Bowland & Shewen 2000). With no current antivirals for livestock on the market, the prospect of using genetic selection for increased response to vaccination would be appealing.

4.1.2 Genetics of BRD

The heritability for resistance to BRD is low ranging from 0.04 to 0.08 ± 0.1 (Snowder et al. 2006; Heringstad et al. 2008). The low heritability would imply that any effort to breed for BRD resistance would require many cattle generations. However, BRD is a complex of diseases and it might be more beneficial to breed for resistance to individual pathogens. There is general agreement that BRSV is probably the most important factor in BRD. There have been many studies investigating the genetics underlying the human form of RSV (HRSV), which

indicate that the host genetics do play a role in resistance to disease caused by HRSV (Janssen et al. 2007).

4.1.3 Bovine respiratory syncytial virus

Bovine respiratory syncytial virus (BRSV) is an enveloped, negative-sense single-stranded RNA *Pneumovirus* of the *Paramyxoviridae* family. It is ubiquitous in both dairy and beef cattle worldwide and causes high morbidity in young animals and is the most important cause of lower respiratory tract infection in young calves (Valarcher & Taylor 2007). The clinical signs of BRSV include severe infection of the lower respiratory tract, resulting in coughing and nasal discharge and abnormal breathing sounds (Antonis et al. 2003). Most of the pathology observed from BRSV infection is not caused by the virus but is due to the host response (Antonis et al. 2003; Glass et al. 2010). Neither natural infection nor vaccination induces long lasting immunity (Meyer, Deplanche, & Schelcher 2008) and young calves are repeatedly infected. It is generally considered that neutralising antibody constitutes the most important protective mechanism against infection, but it is likely that cell mediated immunity also plays a role. A further problem is that waning maternal antibody, which leaves calves at risk of this endemic pathogen, also inhibits induction of protection.

Currently the control of BRSV infection is through management and vaccination programmes of calves using modified live virus (MLV) or killed virus vaccines (Meyer, Deplanche, & Schelcher 2008). In humans no RSV vaccines are licensed due to severe lung pathology caused by host response to an inactivated virus vaccine (Hussell et al. 1998; Meyer, Deplanche, & Schelcher 2008). Although there are cases

of inactivated BRSV vaccines causing pathology in calves (Gershwin 2007), mainly these type of vaccines are considered safe for cattle (Meyer, Deplanche, & Schelcher 2008). In the study described in this chapter, the animals were vaccinated with Rispoval RS (Pfizer), a live attenuated virus vaccine. It is based on BRSV strain RB-94 (a 1969 Belgian isolate) and has been used widely in Europe as a vaccine since 1978 (Zygraich 1982).

4.1.4 Bovine parainfluenza-3 virus

Bovine parainfluenza-3 virus (PIV3) is a single-stranded RNA virus which is closely related to the human parainfluenza virus 3 (HPIV3) and is a member of the *Paramyxoviridae* family (Kapil & Basaraba 1997). Clinical signs of the disease can vary considerably but usually include acute respiratory illness, fever, and malaise (Horwood, Gravel, & Mahony 2008). Whilst PIV3 rarely causes death, it does provide opportunities for secondary infections especially when animals are under stress (Horwood, Gravel, & Mahony 2008). PIV3 is very prevalent in European herds with an estimated 70-85% of animals infected by 9 months of age (Stott et al. 1980).

The protective mechanism against PIV3 infection is primarily humoral although cellular mechanisms are also stimulated (Hussain & Mohanty 1984), with nasal antibody titres providing an accurate measurement of host resistance (Frank & Marshall 1971).

Current management of infection is through intranasal vaccination. The vaccine used in this study was Imuresp (Pfizer) which consists of a live attenuated strain of PIV3

(ts RLB 103) and a live attenuated strain of IBR (ts RLB 106). This vaccine has been shown experimentally to provide significant levels of protection (Bryson et al. 1999).

The role of the host genetics in influencing the levels of protection post-PIV3 vaccination has not been thoroughly explored, however the study by O'Neill indicated a strong sire-based genetic effect (O'Neill 2006).

4.1.5 Bovine herpes virus 1

Bovine herpes virus 1 (BHV1) is a double-stranded DNA virus and a member of the *alphaherpesvirinae* subfamily and is the cause of infectious bovine rhinotracheitis (IBR) and infectious vulvovaginitis/balanoposthitis (Yates 1982). As with all alpha viruses, the herpes virus enters a latent state within the ganglionic neurons after infection (Lemaire et al. 2000), which results in the re-activation of BHV1 under stressful situations.

Infection with BHV1 can result in immunosuppression with the down regulation of interferon (Henderson et al. 2004), apoptosis of CD4+ T cells (Winkler, Doster, & Jones 1999), and reduced expression of MHC Class I and II molecules (Hinkley et al. 2000; Koppers-Lalic et al. 2008).

BHV1 is a common pathogen amongst cattle with an estimated seroprevalence of 42.5% in UK herds (Woodbine et al. 2009). Some countries have eradicated BHV1 through culling of infected animals but most have chosen less extreme measures, instead choosing to vaccinate using either live attenuated or inactivated vaccines (Denis et al. 1996). An effective vaccine against BHV1 should not only protect the animal against infection but also protect from the establishment of latency (Gogev et al. 2002).

Vaccination leads to long lasting immunity (Burroughs et al. 1982). A study into the humoral response of cattle to the vaccination was completed at the Roslin Institute (O'Neill 2006). This study showed that there is a large amount of maternal antibody passed to the calves presumably via the colostrum, since antibodies cannot cross the placenta to the developing calf during pregnancy. This circulating maternal antibody can have an effect on the resultant protection that the vaccine should elicit.

4.1.6 Vaccination

Any vaccination program needs to induce long lasting immunity to infection, although the exact requirements for this are still unknown. The main quantitative measurement of vaccine efficacy is generally the induction of antibodies. However the mechanisms of protection and protective correlates are still being debated. It is highly probable that a substantial T cell response would also be required to induce a long lasting immune response. Indeed for the resolution of BRSV infection the correct balance of CD4+ and CD8+ T cell subsets are required (Taylor et al. 1995; Gaddum et al. 1996; Fogg et al. 2001) and for BHV1 the response of CD8+ cells is considered to be more relevant (Zatechka et al. 1999).

Another major difficulty in the induction of immunity in young animals is the persistence of maternal antibodies (Siegrist 2003). Respiratory infections are a major problem for young cattle as for young humans. There is a limited window in which maternal antibodies protect young animals before maternal antibody wanes. However, the maternal IgG levels have been shown to affect the efficacy of vaccination in humans (Glezen 2003) and in cattle (Vangeel et al. 2005). The levels of pre-existing maternal antibody appear to have had an impact on the vaccination of

the RoBoGen herd, with a decline of BRSV IgG prior to vaccination (O'Neill et al. 2006).

4.1.7 Genetics and vaccination

Understanding the underlying host genetics may help in the design of more efficacious vaccines or lead to selective breeding programs. There is evidence that the host genetics can have an impact on the resultant immune response to vaccination in humans (Glass 2004; Poland et al. 2007). It is thus very likely that the bovine immune response to vaccination will also have a genetic element (Newman et al. 1996). The study by O'Neill (2006) suggested that the response to the respiratory vaccines does have a genetic element, although the precise genes have not been identified. A follow-up study has revealed a number of quantitative trait loci (QTL) for the response to BRSV vaccination suggesting that the variation in response is under multigenic control (Leach and Glass, unpublished observations). BRD has a large economic impact on the livestock industry and vaccination is at best only partially successful in preventing infection and ensuing disease. Thus it would be highly beneficial to identify genes that may play a role in host variation in the response to vaccination, as this might suggest ways to improve vaccine efficacy and perhaps reveal genes playing a role in resistance and susceptibility to disease.

4.1.8 *DRB3* and vaccination

Few studies have endeavoured to study the associations between the BoLA alleles and vaccination (reviewed by Glass (2004)). However, the experimental design of the RoBoGen herd provided an opportunity to investigate the genetics underlying the response after vaccination (Glass 2004; O'Neill 2006).

As the classical MHC gene products are intimately involved in the activation of the adaptive immune response and variation in many humoral responses to viruses, they are highly likely to be, at least in part, responsible for the outcome to vaccination. The MHC locus has also been linked to the humoral response to pathogens in other species, e.g. the chicken MHC has been linked to antibody response to Marek's disease (Bacon, Witter, & Silva 2001) and in humans the HLA genes have been associated with human immunodeficiency virus (HIV) infection outcome (Kaur & Mehra 2009). Whilst the *DRB3* gene has not been associated with the response to any commercial virus vaccines in cattle, they have been associated with the humoral and cellular response to a FMDV peptide (Garcia-Briones et al. 2000) and to a tick vaccine (Sitte et al. 2002) (see also **Chapter 1** Table 1.1). BoLA haplotypes have also been associated with the response to natural BRSV infection (Schrijver et al. 1997). There have been associations noted in humans with the alleles of the DRB gene and response to vaccination (Vidan-Jeras et al. 2000; Gelder et al. 2002).

4.1.9 *Staphylococcus aureus*

Staph. aureus is a gram–positive bacterium which is common in the environment. It is known to cause a wide variety of infections in mammals although symptomatic infection is uncommon. In cattle mastitis is caused by a variety of bacteria with a

major causative agent being *Staph. aureus*. Mastitis in livestock is a serious infection of the mammary gland which affects lactating cattle leading to low milk yield as well as causing severe welfare issues. It results in considerable economic losses estimated to be around 150 to 300 Euros per diseased cow (Davies et al. 2009).

Staph. aureus causes a chronic and recurrent infection which results in the reduction in both the quantity and the quality of milk (Wilson, Gonzalez, & Das 1997). The main immune mechanism against *Staph. aureus* infection is phagocytosis mainly through the action of neutrophils and macrophages (Paape et al. 1981). Unfortunately the inability of these cells to resolve infection is primarily due to a change in morphology and reduction of phagocytosis after the ingestion of caseins and milk fats (Paape & Guidry 1977). However, lymphocytes also play an important role in the clearance of infection (reviewed by Sordillo & Streicher (2002)). The data on the proliferation of PBMC, as measured by Young (2005), give an indication of the host immune response to *Staph. aureus*. A correlation has been demonstrated between the ability of the PBMC to proliferate in response to *Staph. aureus in vitro* (a measure of innate immune response) and lower somatic cell counts (SCC) (Fitzpatrick et al. 1999).

Currently commercial vaccines are available for the prevention of *Staph. aureus* infection. However, the immune response elicited is very variable and they have limited capacity to prevent new infection (reviewed by Talbot & Lacasse (2005)). An ideal vaccine for *Staph. aureus* would likely require the ability to induce a long lasting and effective humoral and cellular immune response.

There have been many QTL studies investigating mastitis resistance/susceptibility, with QTLs discovered on almost all the bovine chromosomes (Davies et al. 2009). Although the heritability of resistance to mastitis is relatively low, it may be possible to use selection to improve the ability to fight infection (Rupp & Boichard 2003). There is considerable on going research into the use of genetic selection and the design of vaccines to prevent mastitis (reviewed by Talbot & Lacasse (2005)).

Many studies have explored the associations between *DRB3* alleles and resistance or susceptibility to mastitis (Table 4.1) (Kelm et al. 1997; Sharif, Mallard, & Sargeant 2000; Park J.Y. et al. 2004; Rupp, Hernandez, & Mallard 2007; Hameed, Sender, & Korwin-Kossakowska 2008; Yoshida et al. 2009). These studies have indicated that there are several *DRB3* alleles which confer either resistance or susceptibility to mastitis. Several groups have suggested that *DRB3.2*23* is associated with resistance to mastitis (Kelm et al. 1997; Dietz et al. 1997; Rupp, Hernandez, & Mallard 2007) and *DRB3.2 *22* is associated with susceptibility (Kulberg et al. 2007; Yoshida et al. 2009).

Sharif et al. (2000) demonstrated that the polymorphisms within the PBC can impact on susceptibility or resistance to *Staph. aureus*. This group found that a glutamine in pocket 4 at position β74 was associated with the occurrence of natural mastitis through *Staphylococcus spp* infection. Within the RoBoGen population, Young et al. (2005) found that sire was a significant factor in the PBMC proliferation in response to *Staph. aureus*. Thus, as the *DRB3* alleles and pockets within the PBC have been associated with mastitis caused by *Staph. aureus,* it would seem likely that this study would also find associations. The BoLA genes have been widely acknowledged as being likely candidates for any breeding program for resistance to mastitis (Table

4.1). Nevertheless, even with a large quantity of data there is contradictory evidence as to which alleles are associated with resistance and/or susceptibility (Table 4.1), most probably due to reasons such as differences in the definition of mastitis, variation in the causative pathogens, cattle breed and the possibility that *DRB3* is not the causative gene but is linked to the responsible gene(s).

***BoLA DRB3* allele**	**Increased/decreased risk of mastitis**	**Reference**
**22[1] (1101)[2]*	• increased risk • decreased risk	Kulberg et al. 2007, Rupp, Hernandez, & Mallard 2007; Yoshida et al. 2009
**11 (0901, 0902, 1202)*	• decreased risk	Rupp, Hernandez, & Mallard 2007; Kulberg et al. 2007
**24 (0101, 0102)*	• decreased risk • increased risk	Rupp, Hernandez, & Mallard 2007; Kulberg et al. 2007 Kelm et al. 1997; Yoshida et al. 2009
**3 (1001, 1002)*	• decreased risk	Rupp, Hernandez, & Mallard 2007, Kelm et al. 1997
**8 (1201)*	• increased risk • decreased risk	Kelm et al. 1997; Rupp, Hernandez, & Mallard 2007 Park et al. 2004
**23 (2701, 2702, 2705, 2706, 2707)*	• decreased risk • increased risk • increased risk	Rupp, Hernandez, & Mallard 2007 Kelm et al. 1997 Sharif et al. 1999 Hameed, Sender, & Korwin-Kossakowska 2008
**16 (1501)*	• decreased risk • increased risk	Kelm et al. 1997 Sharif et al. 1999; Park et al. 2004; Yoshida et al. 2009

[1]*BoLA DRB3* alleles shown as RFLP nomenclature; [2] the IPD (immuno polymorphism database) nomenclature (http://www.ebi.ac.uk/ipd/mhc/bola/nomen_rules.html).

Table 4.1 *BoLA DRB3* alleles identified as having a significant association with mastitis and/or somatic cell counts (SCC).

4.2 Aims

This chapter aims to investigate the association between the immune response (IgG levels) to BRSV, BHV1 and PIV3 after vaccination and the alleles and PBC of *BoLA DRB3*. In addition, the associations between the alleles of *DRB3* and the PBMC proliferation to *Staph. aureus* and to the mitogen phytohaemagglutinin (PHA) have been investigated.

4.3 Materials and methods

All vaccination procedures, bleeding and assays were carried out by staff at The Roslin Institute.

4.3.10 Animals

The study population is the same as stated in **Chapter 2**. The 12 sires were used to produce 3 lines of F1 x F1 (N=255), Charolais x F1 (N=67) and Holstein x F1 (N=82) giving a total of 407 animals (female=201, male=206). The animals were born and sampled over 3 years in 3 separate cohorts from 1999 to 2001. The age of the calves when immunised with the vaccines (see below) ranged from 60 to 167 days. All of the animals received colostrum before 6 hours of age. The male calves had unrestricted access to their dams until approximately 4 months of age. The female calves were weaned by 36 hours and were raised indoors on milk-replacement followed by a propriety compound diet. For the 77 day duration of this study all the animals were housed in single sex groups (O'Neill et al. 2006).

4.3.11 Vaccination protocol of animals

Each animal was immunised on day 0 of the study with the attenuated BHV1 and PIV3 vaccine, Imuresp RP (Pfizer Animal Health, Surrey, UK), according to the manufacturer's instructions. Vaccine which had been rehydrated was administered intranasally in a 2 ml dose, half of which was placed in each nostril. (O'Neill 2006) Each animal also received 2 ml of Rispoval RS (Pfizer Animal Health, Surrey, UK), an attenuated live vaccine intramuscularly, according to the manufacturer's recommendations, on day 28 following vaccination with Imuresp, and a boost intramuscularly was given 21 days after the first dose on day 42.

4.3.12 Sampling for PBMC proliferation to *Staph. aureus*

Both males and female calves were used for the analysis of the PBMC proliferation response to *Staph. aureus* (male n=130 and female n=138) which gave a total study population of n= 268 from 2 cohorts (Young et al. 2005).

4.3.13 Sampling for vaccine responses

Blood samples were collected by jugular venipuncture on days 0, 14, 28, 42, 63 and 77 following vaccination. Serum was collected within 2 hours of sampling and stored at −20°C until testing (O'Neill 2006).

4.3.14 Vaccine ELISA

The ELISA analysis was carried out by Dr Ronan O'Neill (O'Neill et al. 2006). The ELISA was performed by Dr Ronan O'Neill (O'Neill 2006).

4.3.14.1 PBMC proliferation assay- for *Staph. aureus* and PHA

The proliferation assay was carried out by Dr. Fiona Young and described in her thesis and publication (Young 2002; Young et al. 2005).

4.3.15 Statistical analysis for vaccine data

The IgG relative optical density (ROD) data for each trait was normalised by Log_e transformation to obtain a normal distribution. A REML model as described in **Chapter 3** was considered the most appropriate for the analysis. The addition of sex as a factor (2 d.f.) and sire as a random effect were added to the model.

Thus the linear model was:

$$y_{ijklmn} = l_i + c_j + u_k + q_n + b_m + MHC_{ijkmn} + a_{ijlmn} + e_{ijlmn}$$

where: y_{ijkl} is the observed phenotypic trait; l_i fixed effect of breed-cross (CB, HB or F2); c_j fixed effect of the cohort (years 1, 2, or 3); b_m fixed effect of sex (male or female); MHC_{ijk} linear regression of the MHC allele/amino acid (0, 1 or 2 copies); u_{ijkl} random effect of the dam; q_{ijkl} random effect of the sire; a_{ijkl} covariate effect of the age of the animals at time of vaccination; e_{ijkl} the residual error. All statistical analysis was performed using Genstat with the significance of fixed effects such as MHC variant assessed with a Wald test. If MHC variants were significant (at least $p<0.05$), then further analysis was conducted by generating predicted means and conducting a Student's t-test using pair wise comparisons (Genstat v9.0 VSN International).

4.3.15.1 Statistical analysis for *Staph. aureus* PBMC proliferation

The PBMC proliferation counts per minute (cpm) were normalised by Log_e transformation to obtain a normal distribution. The REML model was the same as above with the added covariate of control proliferation and without animal age as a factor. If MHC variants were significant (at least $p<0.05$), then further analysis was conducted by generating predicted means and conducting a Student's t-test using pair wise comparisons.

4.4 Results

4.4.1 Animal information

The animals were all typed at the *DRB3* locus as described in **Chapter 2.** A total of 407 animals were used in the analysis of the IgG vaccine responses and 287 animals were used for the PBMC proliferation response. Data were normalised by Log_e transformation for all datasets. An analysis of the fixed and random factors for the BRSV vaccine response and the PBMC proliferation to *Staph. aureus* in the RoBoGen herd has already been published by others (O'Neill et al. 2006) and (Young et al. 2005). In addition, similar analyses were carried out by O'Neill (2006) for the PIV3 and BHV1 data sets. Although genetic and sire effects were observed, no previous research has attempted to identify any candidate genes (or chromosomal loci) for these data sets. Thus the data analysis presented in this chapter is new and novel.

4.4.2 BRSV dataset

The mean, median, and quartiles for the IgG response to BRSV vaccination, as measured by a commercial ELISA, have been published in O'Neill et al (2006) and there was a high degree of variation across all time points, which is partially accounted for by sire and breed cross as well as environmental factors including maternal antibodies and differences in management between sexes (O'Neill et al. 2006). Overall, there was a decrease in IgG levels from day -28 to day 0 (day of vaccination) most likely due to the decline in maternally derived antibodies (O'Neill et al. 2006). There was an increase in IgG levels post-vaccination until a peak IgG response was attained at day 35. The mean ROD percentages for days -28, -14, 0, 14, 35 and 49 were 27.10, 19.99, 13.79, 20.00, 38.94 and 31.20% ROD, respectively (O'Neill 2006). The IgG levels significantly increased across time ($p<0.001$).

4.4.3 Effect of *DRB3* alleles on the IgG immune response to BRSV vaccine

The frequency of the 18 different *DRB3* alleles can be found in **Chapter 2**. The mean median and upper and lower quartiles were examined for the peak day (day at which highest % ROD was observed) for each *DRB3* allele (Table 4.2). The peak day was on day 35 for the majority of alleles, with the exception of **1801*, **14011* and **3201* where the peak day was on day 49 post-vaccination. The highest mean allele was **0801* (61.46% ROD) but this allele is represented only once in the herd. The second highest allele was **1501* (55.81% ROD) which is still a low frequency allele. The allele with the lowest mean level was **3201* (27.60% ROD).

Alleles **0901*, **1002* and **1701* had significant p values post-BRSV vaccination ($p<0.05$). *DRB3*0901* was significant at day 14 post-vaccination, while **1002* and **1701* were both significant at day 49 post-vaccination. Only allele **0801* was significant pre-vaccination at days -28, -14 and 0 ($p<0.05$).

However, alleles **1002*, **1701* and **0801* were at very low frequencies within the herd (<10%), and the significant p value generated from *DRB3*0901* is due to a single homozygous animal.

***DRB3* alleles**	**Number of animals with allele**	**Mean**	**Median**	**Lower Quartile (Q1)**	**Upper Quartile (Q2)**
**0801*	1	61.46	61.46	61.46	61.46
**1501*	7	55.81	58.91	42.96	71.94
**1102*	2	50.41	50.41	37.57	63.25
**1801#*	6	44.41	39.88	24.93	74.63
**2002*	9	44.32	42.75	27.05	52.75
**1101*	124	43.07	42.09	31.58	54.36
**1701*	10	41.90	39.40	29.98	61.78
**0901*	53	41.68	41.22	27.11	52.37
**0501*	28	41.15	35.84	26.31	55.56
**0701*	19	40.17	46.21	21.16	54.58
**0201*	49	40.00	37.82	30.23	49.17
**2703*	9	39.94	34.97	27.28	54.67
**1001*	36	39.69	39.50	19.90	53.12
**1601*	95	38.40	37.04	22.38	50.09
**2707*	156	37.14	34.31	19.27	51.43
**0101*	56	36.91	37.59	24.60	48.06
**14011#*	2	36.61	36.61	26.00	47.23
**1201*	45	35.97	33.21	22.05	47.79
**0601*	42	35.53	33.32	21.10	45.58
**0902*	17	32.73	22.29	17.03	46.51
**1002*	1	31.62	36.62	36.62	36.62
**3201#*	3	27.60	14.00	11.53	47.06
Overall	407	38.94	37.45	23.58	51.21

 # Day 49 peak day

Q1 and Q2 represent the lower and upper quartiles, respectively.

Table 4.2 *DRB3* alleles in the population: mean, median, upper and lower quartiles at peak day (Day 35) for BRSV IgG (% ROD).

4.4.4 Effect of binding pockets on response to BRSV vaccine

There are several positions within the PBC which have a significant association with the IgG response to BRSV. Pocket 7 positions β28 and β30 are both significant at day 14 post-vaccination ($p<0.05$). Pocket 9 β37 is significantly associated before vaccination at day -28 ($p=0.036$).

Pocket 4 β13 is significant at day 0 ($p<0.05$) and β70 at day 49. Further REML analysis revealed that an asparagine (N) at position β74 within pocket 4 is highly significant at day 14 ($p<0.001$) and had p values <0.05 for days 35 and 49 post-vaccination. Further analysis revealed that animals homozygous for N at this position (n=4) had a lower mean IgG ROD % before vaccination (although not significant) but the antibody levels increased to the same levels as those in animals expressing a single N at β74 after the boost on day 21 (Figure 4.1). At day 49, animals without an N at this position had highly significantly greater IgG levels to the BRSV vaccine compared to those expressing at least one N ($p<0.001$ obtained by Student's t test using 0N and 2N predicted means) (Figure 4.1).

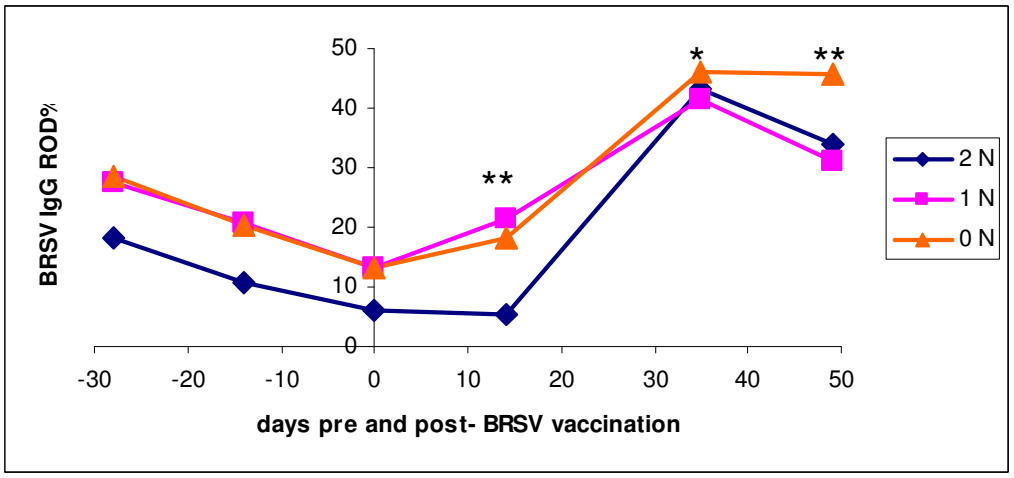


Figure 4.1 BRSV IgG % ROD pre- and post-BRSV vaccination with position β74: homozygous for asparagine (N): ◆; heterozygous for N: ■; no copies of N: ▲. p values determined by Student's t-test from predicted means: * p<0.01, **p<0.001. The homozygous predicted means were used in Student's t-test for significant values.

4.4.5 PIV3 and BHV1 datasets

The mean, median, and quartiles for the IgG response to vaccination with Imuresp against PIV3, as measured by a commercial ELISA, have been reported in O'Neill (2006) and there was a high degree of variation across all time points which is partially accounted for by sire and environmental factors (O'Neill 2006). The PIV3 response mean values for days 0, 14, 28, 42, 63 and 77 were 38.63, 36.17, 42.10, 46.63, 52.62 and 55.96% ROD respectively. The PIV3 IgG levels showed a steady increase across the time points post-vaccination which is not significant across time points (Figure 4.2).

The BHV1 mean values for days 0, 14, 28, 42, 63 and 77 were 34.60, 27.01, 22.83, 18.58, 14.40 and 11.71% ROD respectively. The BHV1 IgG levels showed a steady decrease in the IgG levels post-vaccination ($p<0.001$) (Figure 4.3). The high levels of BHV1 antibody at day 0 are likely to be maternally derived and the subsequent levels of IgG post-vaccination do not show any convincing response.

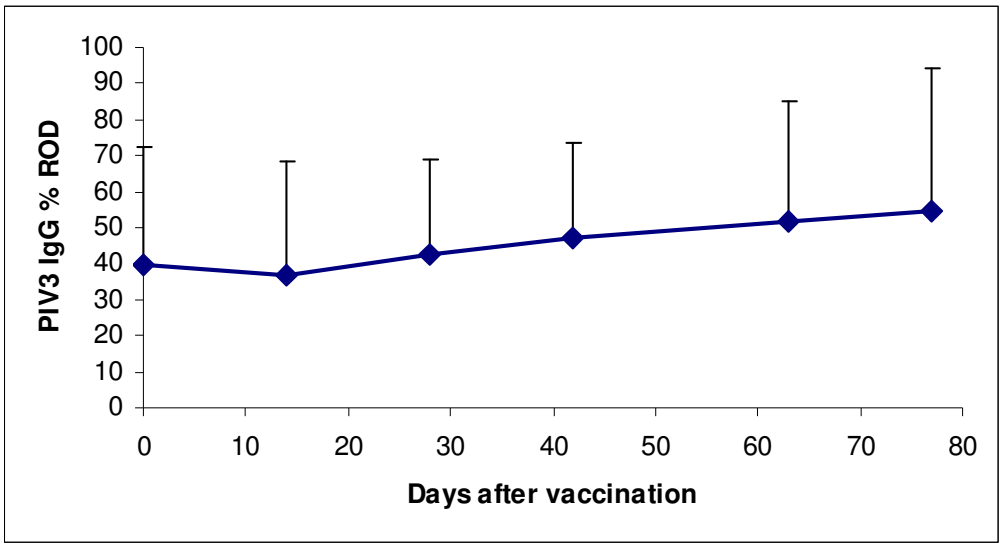


Figure 4.2 PIV3 IgG (%ROD) post-vaccination levels of all animals as measured by ELISA (using data from O'Neill 2006). Mean ± SD.

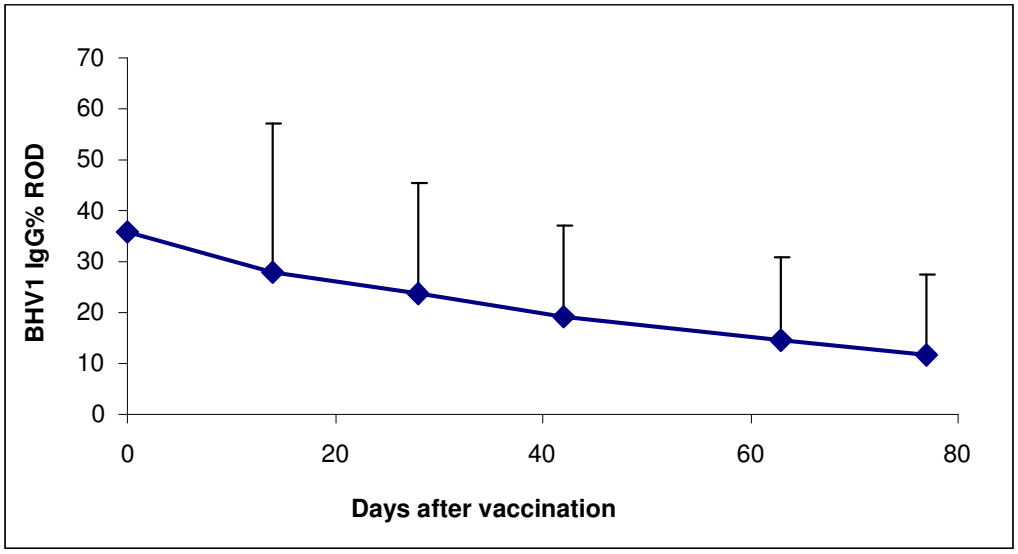


Figure 4.3 BHV1 IgG (%ROD) post-vaccination levels of all animals as measured by ELISA (using data from O'Neill 2006). Mean ± SD.

4.4.6 Effect of *DRB3* alleles on IgG levels PIV3 and BHV1 IgG levels

From the REML analysis there were no *DRB3* alleles significant for the PIV3 IgG levels (results not shown). There were however several alleles which were significant for the BHV1 IgG levels: *DRB3 *0201* and **0901* were significant at day 63, **1601* at day 42 and **0701* at day 77 ($p<0.05$). As these were only significant at single time points they were not analysed further. *DRB3*1701* was significant at days 14 and 63 and was analysed further (Figure 4.4). Animals positive for one copy of **1701* had significantly higher levels of IgG for BHV1 ($p<0.05$).

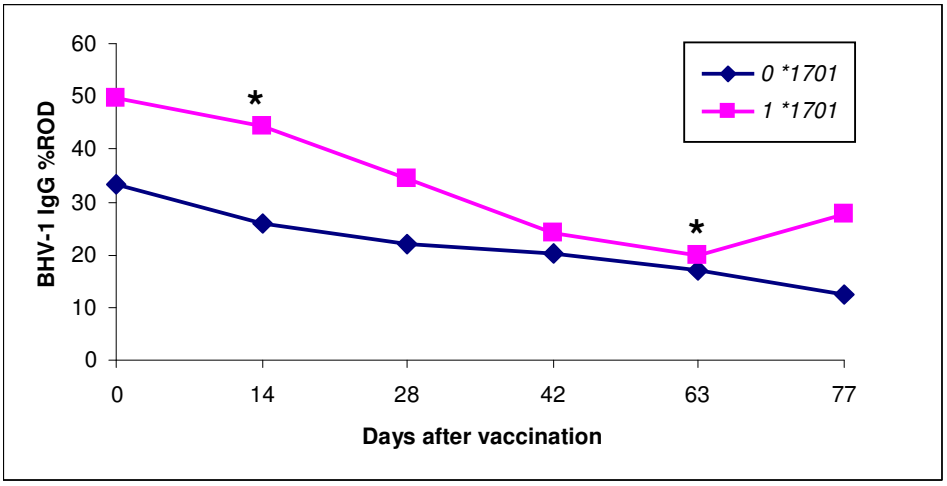


Figure 4.4 BHV1 IgG levels (%ROD) with 1 copy of **1701* or with no copies of **1701*. p values determined by Student's t-test from predicted means: *p<0.01, **p<0.001.

4.4.7 Effect of binding pockets on PIV3 and BHV1 IgG levels

As in **Chapter 3**, all animals were subdivided according to the PBC pockets and results fitted in the REML model. The amino acids in each pocket position for each allele are shown in **Chapter 2**.

4.4.7.1PIV3

For the PIV3-specific IgG levels, pocket 7 had two positions which were significant from the REML analysis, β61 ($p<0.001$) at days 28 and 42 post-vaccination and β67 ($p<0.05$) at day 28. Glutamic acid (E) and arginine (R) at β70 within pocket 4 were both significantly associated with PIV3-specific IgG at day 28 ($p<0.05$). Two copies of E at β70 were associated with higher IgG levels whilst 2 copies of R were associated with lower levels of IgG to PIV3 vaccination (Figure 4.5). The two homozygote predicted means values were used in the Student's t-test calculations.

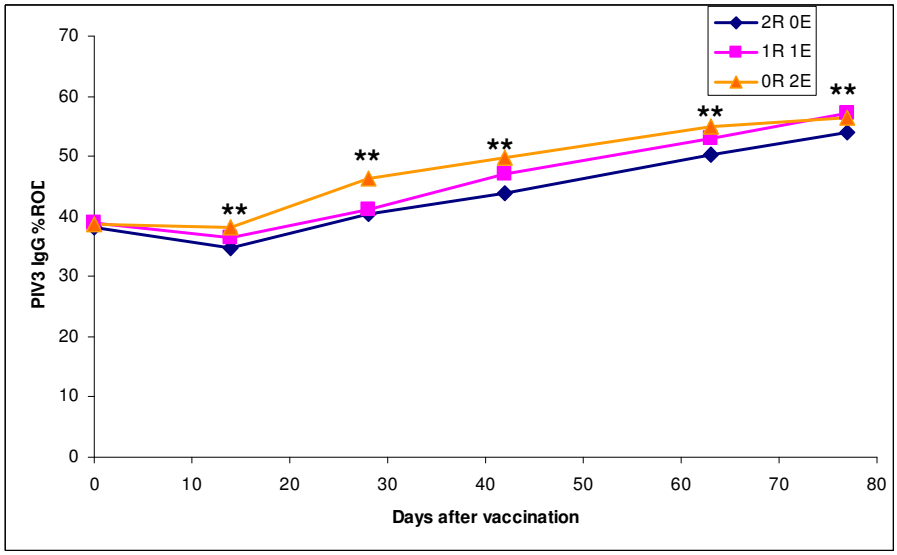


Figure 4.5 Pocket 4 position β70. (a) anti-PIV3 IgG1 responses position β70 homozygous for glutamic acid (E) ▲, heterozygous for E and R■, or homozygous for arginine (R) ♦. p values determined by Student's t-test from predicted means: **p<0.001, *<0.05. The homozygote predicted means values were used in the Student's t-test.

4.4.7.2BHV1

The BHV1 IgG levels were significantly associated ($p<0.05$) with pocket 6 position β11 at day 77 post-vaccination, pocket 7 position β67 at days 0 and 14 and pocket 9

positions β37 at day 28 and β57 at day 0. Pocket 4/7 position β71 was also significant at day 77.

4.4.8 Staph. aureus results

At day 4 following culture, the PBMC proliferative response to *Staph. aureus* was 23235.72 (cpm) and for response to PHA was 27187.14 (cpm). There was considerable variation observed for both the proliferation to *Staph. aureus* and PHA, which was partially accounted for by breed and sire effects (Young 2002).

4.4.9 Effect of *DRB3* alleles on proliferation to *Staph. aureus* and PHA

Allele **1701* (n=6) was highly significant ($p<0.001$) for the PBMC proliferation to *Staph. aureus*, with the presence of the allele resulting in a higher level of proliferation. Allele **0601* was also significant ($p=0.05$), where a single copy of the allele resulted in a higher level of proliferation compared to no copies or 2 copies (n=4). Other alleles worth noting are **2703* and **2707* which showed suggestive associations ($p=0.09$ and 0.07 respectively).

Alleles **1601* and **1701* were both significant ($p<0.05$) for the response to PHA, with the presence of **1601* resulting in lower levels of proliferation and **1701* resulting in higher levels.

4.4.10 Effect of binding pockets on proliferation to *Staph. aureus* and PHA

For the response to *Staph. aureus*, 4 positions within the PBC were significant: pocket 6 position β11 ($p=0.032$), pocket 7 position β28 ($p=0.05$), position 61 which is part of both pocket 7 and pocket 9 ($p=0.02$) and pocket 4 position β71 ($p<0.05$). Animals which expressed an E at position β71 had a higher level of PBMC proliferation.

There were no significant positions in the PBC for the response to PHA.

4.5 Comparison of associations with *DRB3* alleles and PBC pocket positions and humoral and cellular response in the RoBoGen herd

In **Chapter 3** and this chapter there are many statistically significant associations with both the *DRB3* alleles and positions within the PBC. To allow a clearer comparison across immunising agents and antibody and cellular responses these results have been organised into tables.

	Immunising agent/day[#]			
Allele	**BRSV**	**BHV-1**	**FMDV15 peptide**	
			IgG1	**IgG2**
0201	N.A.	63	N.A.	N.A.
0701	N.A.	77	70	N.A.
0801	-28; -14; 0	N.A.	N.A.	N.A.
0901	14	63	14; 56	N.A.
0902	N.A.	N.A.	56;	N.A.
1001	N.A.	N.A.	56; 70	14; 56; 70 (higher IgG2)
1002	49	N.A.	N.A.	N.A.
1601	N.A.	42	14; 28; 56;70	14; 28; 56; 70 (lower IgG2)
1701	49	14; 63	N.P.	N.P.
2707	N.A.	N.A.	14; 56	14 (lower IgG2)

[#]day following first immunisation with agent is shown in Table cells. Shading denotes a significant statistical association, N.P. not present. N.A. no association.

Table 4.3 Comparison of the statistically significant effects of the *DRB3* alleles on the antibody responses to different immunising agents in the RoBoGen herd: vaccines for BRSV and BHV-1, and FMDV peptide. Note no alleles were significantly associated with anti-PIV-3.

	Stimulus/day[#]					
			FMDV peptide		ConA	
Allele	***Staph. Aureus*** **(Day 4 of culture)**	**PHA**	**T cell**	**IFN-γ**	**T cell**	**IFN-γ**
****0101***	N.A.	N.A.	28	N.A.	N.A.	28
****0501***	N.A.	N.A.	0	N.A.	N.A.	28
****0601***	higher heterozygote	N.A.	N.A.	N.A.	N.A.	N.A.
****0902***	N.A.	N.A.	56; 70	N.A.	N.A.	N.A.
****1001***	N.A.	N.A.	56; 70	N.A.	N.A.	N.A.
****1101***	N.A.	N.A.	0	28;70 low/non responder	N.A.	N.A.
****1201***	N.A.	N.A.	56; 70 Non/low responder	N.A.	0; 28; 56; 70	N.A.
****1601***	N.A.	lower response	N.A.	N.A.	N.A.	N.A.
****1701***	higher response	higher response	N.P.	N.P.	N.P.	N.P.
****2703***	Suggestive	N.A.	N.A.	Non/low responder; 28;70	N.A.	N.A.
****2707***	Suggestive	N.A.	N.A.	70	N.A.	28

[#]day following first immunisation with agent is shown in Table cells. Shading denotes a significant statistical or a statistically suggestive association, N.P. not present. N.A. no association.

Table 4.4 Comparison of the significant effects of the *DRB3* alleles on the cell mediated responses to different stimuli in the RoBoGen herd: FMDV peptide, *Staph. aureus* and ConA. No significant pocket effects were detected with PHA.

		Immunising agent/day relative to immunisation				
Pocket	**Amino acid Position**	**BRSV**	**PIV-3**	**BHV-1**	**FMDV peptide (IgG1)**	**FMDV peptide (IgG2)**
1	**86**	N.A.	N.A.	N.A.	70	28; 56
4	**13**	0	28	N.A.	N.A.	28; 56
	70	49 (E<R)[$]	28 (E>R)[$]	N.A.	14 (E<R)[$]	14; 28; 56 (E<R)[$]
	74	14; 35; 49	N.A.	N.A.	56	N.A.
4/7	**71**	N.A.	N.A.	77	N.A.	14
6	**11**	N.A.	N.A.	N.A.	14;	14
7	**28**	14	N.A.	N.A.	N.A.	28
	30	14	N.A.	N.A.	N.A.	14
	67	N.A.	28	0; 14	N.A.	N.A.
7/9	**61**	N.A.	28; 42	N.A.	14; 56; 70	14; 56; 70
9	**37**	-28	N.A.	28	14; 56 70	14
	57	N.A.	N.A.	0	14;	14; 28; 56; 70
	60	N.A.	N.A.	N.A.	N.A.	14; 56
10	**56**	N.A.	N.A.	77	N.A.	14; 28; 56; 70

[#]day following first immunisation with agent is shown in Table cells [$]animals expressing glutamic acid (E) had significantly greater (>) or lower (<) responses than animals expressing arginine (R) at this position. Shading denotes a significant statistical association, N.A. no association.

Table 4.5 Comparison of the effects of the PBC pockets of DRB3 on the antibody responses to different immunising agents in the RoBoGen herd: vaccines for BRSV, PIV-3 and BHV-1, and FMDV peptide.

		Stimulus/day[#]				
Pocket	**Amino acid Position**	***Staph. aureus*** **(Day 4 of culture)**	**FMDV peptide**		**Con A**	
			T cell	**IFN-γ**	**T cell**	**IFN-γ**
1	86	N.A.	N.A.	28	N.A.	70
4	13	N.A.	N.A.	N.A.	0; 70	56
	70	N.A.	N.A.	28; 70	0; 56	56
	74	N.A.	N.A.	28; 70	N.A.	N.A.
4/7	71	√ (E<R)[$]	56 (E>R)[$]	28; 70 (E>R)[$]	0 (E<R)[$]	N.A.
6	11	√	N.A.	56	N.A.	56
7	28	√	56	56; 70	56; 70	N.A.
	30	N.A.	N.A.	70	N.A.	70
	47	N.A.	N.A.	28	N.A.	N.A.
	67	N.A.	56; 70	70	56	N.A.
7/9	61	√	56; 70	70	N.A.	70
9	9	N.A.	N.A.	28	N.A.	56; 70
	37	N.A.	56	28; 56	N.A.	56
	57	N.A.	70	28; 56; 70	N.A.	28; 56; 70
	60	N.A.	N.A.	70	N.A.	70
10	56	N.A.	N.A.	28; 56; 70	N.A.	N.A.

[#]day following first immunisation with agent is shown in Table cells: [$]animals expressing glutamic acid (E) had significantly greater responses than animals expressing arginine (R) at this position. Shading denotes a significant statistical association, N.A. no association.

Table 4.6 Comparison of the effects of the PBC pockets of *DRB3* on the cell mediated responses to different stimuli in the RoBoGen herd: FMDV peptide, *Staph. aureus* and ConA. No significant pocket effects were detected with PHA.

4.6 Discussion

In this chapter the same female animals which were studied in **Chapter 3,** together with many of the second generation males, were analysed for *DRB3* allele associations with their humoral response to several additional stimuli, including two vaccines, Rispoval against BRSV and Imuresp against BHV1 and PIV3, as well as cellular responses to a mastitis causing pathogen *Staph. aureus* and a mitogen, PHA. Considerable variation was seen in the response to all pathogens, some of which has been accounted for by genetic (sire and breed-cross) and environmental factors (cohort, age, sex, maternal antibody levels) (Young 2002; Young et al. 2005; O'Neill et al. 2006; O'Neill 2006). Although there are no previous reports on associations of *BoLA DRB3* alleles and responses to viral vaccines in cattle, a number of studies suggested that it would be likely that significant associations would be seen between *BoLA DRB3* alleles and the responses to the respiratory viral vaccines and to the mastitis-causing pathogen *Staph. aureus*. HLA alleles have been associated with efficacy of vaccination against viral pathogens in humans, most notably in Hepatitis B and measles vaccination (Blackwell, Jamieson, & Burgner 2009) and cattle responses to vaccination have also been associated with alleles of the BoLA region (Newman et al. 1996; Sitte et al. 2002). Furthermore many associations with BoLA Class II alleles were found for the response to an FMDV peptide in **Chapter 3** (Baxter et al. 2009) and a study on the antibody response to epitopes present in BRSV found that there were associations with either high or low response depending on BoLA haplotypes after natural infection (Schrijver et al. 1997). Additionally, there have been many studies on mastitis in cattle that show significant associations

with BoLA *DRB3* alleles (Table 4.1) (Kelm et al. 1997; Sharif, Mallard, & Sargeant 2000; Park J.Y. et al. 2004; Rupp, Hernandez, & Mallard 2007; Hameed, Sender, & Korwin-Kossakowska 2008; Yoshida et al. 2009) and the PBMC proliferation assay to *Staph. aureus* in young male calves may be predictive of the likelihood of mastitis in adulthood in their daughters (Fitzpatrick et al. 1999). Taken together these studies supported the hypothesis that variation in the responses measured in this Chapter would be related to *BoLA DRB3* polymorphisms. However, despite the increase in animal numbers, fewer significant associations were found compared to **Chapter 3**. Seven *DRB3* alleles were discovered to be significantly associated with the IgG response to vaccination. The PBMC proliferation response was significantly associated with four alleles for *Staph. aureus* and two alleles for PHA. Even when the animals were subdivided into pocket positions, fewer associations were observed compared to the FMDV15 peptide responses. There are many possible explanations for this including some technical reasons and these are discussed below.

One possible reason for the differences between the studies may be due to differences between the assays used and the read-outs. The FMDV ELISAs for IgG1 and IgG2 were set up so that precise concentrations of specific antibody could be measured (see **Chapter 3)** whereas the vaccine ELISAs were commercial assays intended for epidemiological surveys of prevalence, and are not designed to measure precise levels in individual animals. In contrast to the FMDV ELISAs where sera were diluted out until the readings fitted onto a standard curve, the vaccine ELISAs utilised fixed dilutions of sera, and compared the readings to a standard control provided with the kit. This has an impact on the sensitivity and accuracy of the PIV3 and BHV1 IgG-specific measurements.

From the results in this chapter it is possible to conclude that the Rispoval vaccine increased the level of circulating BRSV-specific IgG post-vaccination. However it is more difficult be confident about the efficacy of the Imuresp vaccine, as the levels of PIV3-specific IgG were not significantly higher after vaccination and the levels of BHV1-specific IgG declined post-vaccination. In both vaccine studies the animals had high levels of pre-existing IgG to all three respiratory pathogens, which is due to either circulating maternal antibody and/or previous exposure to these pathogens. It is highly probable that the calves had already been exposed to the viruses prior to vaccination as they are endemic within British herds (Stott et al. 1980; Woodbine et al. 2009), although none of the animals had any obvious clinical signs.

In humans the persistence of circulating maternal antibodies has an effect on the efficacy of vaccination against several important infectious diseases (Siegrist 2003). For example, one of the difficulties in designing an effective vaccine to human RSV is the need to vaccinate young infants who are compromised by the presence of maternal antibodies (Glezen 2003). A similar scenario exists for young calves, whereby it is considered preferable to vaccinate at an early age against BRSV (because it is endemic) yet maternal antibodies affect vaccine efficacy (Zygraich 1982; Kimman, Westenbrink, & Straver 1989). In cattle maternal antibodies interfere with the humoral response to vaccination against BVDV, whereas a T cell mediated response is not affected (Endsley et al. 2003). In the present study the levels of anti-BRSV IgG were declining prior to vaccination, which is most likely due to the reduction in the levels of circulating maternal antibody (O'Neill et al. 2006; O'Neill 2006).

There is no reported study into the genetic influence on the clearance of maternal antibody in cattle. However, the transfer of IgG from dam to calf via the colostrum has high heritability (Norman, Hohenboken, & Kelley 1981). This suggests a genetic component influenced by both the dam and calf. Polymorphisms in the Fc receptor α-chain gene have been associated with the levels of IgG in newborn calves (Laegreid et al. 2002). Here there is evidence that there is pre-existing IgG to all three respiratory pathogens. If clearance of maternal antibody is under different genetic control than vaccine responses then it is possible that this too could help explain the observation that fewer significant *BoLA DRB3* alleles were observed post-vaccination compared to the FMDV peptide results in **Chapter 3**, where all of the animals were naïve to the peptide.

The *Staph. aureus* assay described in this chapter is likely measuring an anamnestic response to the bacteria, as a naïve response would have peaked at day 7 (not day 4) (Young 2002). As any previous exposure to mastitis pathogens is an unknown factor in the current study, this makes it more difficult to accurately discern any MHC effects. In addition this assay has only been reported in one study to have associations with susceptibility to mastitis (Fitzpatrick et al. 1999) and would need further validation in other populations.

Fewer significant alleles were seen in association with the vaccine responses compared to the responses to the FMDV peptide (**Chapter 3**), with no significant alleles at all for response to PIV3 vaccination (summarized in Table 4.3). This is not unexpected as these vaccines contain whole virus particles and therefore many more

epitopes, which would decrease the chance that a single allele would be wholly responsible for the IgG response. The same consideration applies for the *Staph. aureus* results, since the bacterium has many more epitopes compared to a 40-mer peptide.

Epitopes have been identified for BRSV, which induce antibody (Taylor et al. 1992) and T cell responses (Fogg et al. 2001). The extent of antibody response to the main BRSV epitope was associated with certain BoLA haplotypes in naturally infected cattle (Schrijver et al. 1997). However, it is interesting to note that Schrijver et al. (1997) noted differences between antibody specificities produced from those animals which were naturally exposed and those which were vaccinated. Epitopes have also been identified for the human form of PIV3 and evidence suggests that a number of these epitopes are shared between human and cattle (Coelingh et al. 1986). To the author's knowledge there are no studies investigating associations between antibody response to bovine PIV3 epitopes and MHC alleles. A number of cytotoxic T-cell epitopes for BHV1 has been described (Zatechka et al. 1999), and epitopes have been identified for antibody response to BHV1 (Chowdhury 1997). Moreover it is possible to use allele-specific peptide motifs for BoLA Class I molecules and identify potential CTL epitope peptides (Hegde & Srikumaran 1996; Hegde et al. 1999). This opens up the possibility of using effective peptide vaccines, and the option of utilizing the information on the DRB3 alleles.

How protection is achieved post-vaccination through the action of antibodies and T cells is still debated (reviewed by Burton (2002)). However, in most vaccine efficacy studies generally only a humoral response is equated with protection, as this is more easily measured, even though cellular responses are likely to play an important role.

Indeed for the protection against *alphaherpesviruses* such as BHV1 the CTL response is perhaps more important than the antibody titre (Zatechka et al. 1999). In this study only virus-specific IgG levels were measured, due in main to logistics, as there was limited availability of appropriate assays, resources or personnel to conduct them. Furthermore, it has proved difficult to detect T cell responses to these vaccines (Taylor et al. 1995; Fogg et al. 2001).

The low numbers of significant alleles observed in this chapter could be because the alleles and polymorphisms in pockets of *DRB3* have a greater effect on the Th1 pathway. In **Chapter 3** and in this chapter, as summarized in tables 4.3, 4.4, 4.5 and 4.6 it is demonstrated that the *DRB3* alleles and pockets were more significantly associated with a Th1 (IgG2 and IFN-γ) response than with IgG1. It is important to consider these implications for vaccine design as for many intracellular pathogens, such as viruses, the protective mechanism is more closely related to a Th1 than a Th2 response (Brown, Rice-Ficht, & Estes 1998). In addition there are differences in the alleles which are associated with IgG1 and IgG2 response (Table 4.4 and **Chapter 3**).

There are not many associations which have been noted between single stimuli and the *DRB3* alleles and some are not highly significant. However, when all the associations are taken together it becomes more convincing that there are significant associations.

This chapter and **Chapter 3** have highlighted *DRB3* alleles which have a significant association with more than one vaccine IgG response; *DRB3*0901*, **0701*, **1601* and **1701* (Table 4.3). Allele **0901* was significant for the response to BHV1, BRSV and in **Chapter 3** to the FMDV peptide. This allele is also suggested to have

an association with reduced SCC (Table 4.1). The **0701* and **1601* alleles were both significant for IgG to BHV1 and FMDV. Finding alleles that confer a higher response to vaccination against many pathogens might provide new targets for breeding programs (Wilkie & Mallard 1999; Gay et al. 2007). Juliarena et al. (2009) suggest that selecting for BLV resistant cattle would not affect the response to several other vaccines, including BHV1 and FMDV. Important considerations would be to ascertain that any breeding programs did not adversely affect other immune responses. For example, it would be important not to diminish the ability of cattle to make protective responses to vaccination nor increase the propensity for pathogenesis in response to vaccination. Furthermore it would also be important to ensure that any breeding programme did not increase susceptibility to infection.

In this study alleles **1701* and **0601* were significantly associated with the PBMC response to *Staph. aureus*. However, neither of these alleles have been associated with mastitis in other studies, nor were any other traits described in this thesis associated with **0601*. However, **1701* was associated with higher levels of PBMC proliferation to both *Staph. aureus* and PHA (Table 4.4).

It is interesting to note that allele **1701* was significant for the response to vaccination against BRSV, BHV1 and the PBMC response to both *Staph. aureus* and PHA. A single copy of **1701* resulted in higher BHV1-specific IgG levels after vaccination, and higher proliferative PBMC response to both *Staph. aureus* and to PHA. However, for the BRSV-specific IgG response a single copy of **1701* resulted in a significantly lower response at day 49. *DRB3*1701* was identified as a possible marker for breeding BLV resistant cattle due to a strong association with low proviral load (Juliarena et al. 2009). Unfortunately the female population used in

Chapter 3 had only two animals positive for the allele **1701* and it was at a low frequency within the whole RoBoGen population; therefore the results should be treated with caution and would need further validation.

Alleles **2703* and **2707* have been previously identified as significantly associated with mastitis, although there is contradictory evidence as to whether these alleles are of benefit or are detrimental to mastitis outcome (Table 4.1). In this chapter, suggestive evidence indicates that these alleles were associated with an increase in the PBMC proliferation to *Staph. aureus*. The *2703 and *2707 alleles differ by only 2 base pairs (**Chapter 2**), and all the alleles starting *270 have near identical nucleotide sequences. The variety in the mastitis pathogen population may result in different alleles providing different levels of protection and hence might explain the contradiction in some populations.

PHA was chosen as a positive control for the stimulation of PBMC in the *Staph. aureus* assay (Young 2002). PHA stimulates non-specific proliferation of T lymphocytes and therefore would be a measure of innate immunity. A study by Dietz et al. (1997) investigating several immune parameters found no significant association of *DRB3* alleles after stimulation with PHA or ConA. Therefore it is interesting to note that in this study a few associations were observed with the *DRB3* alleles and the PBC pockets. In **Chapter 3** ConA was used as a positive control in the stimulation of cell division for T cells and production of IFN-γ. The results from **Chapter 3** (Table 4.4) showed that there were many alleles significantly associated with IFN-γ production after stimulation with ConA.

There is no single allele which is a marker for general disease resistance, although there are several alleles which appear to be significant in both the humoral and cellular response (Tables 4.3 and 4.4). Allele **1701* is significant for the antibody responses to BRSV and BHV-1 as well as being significant for higher PBMC proliferation to both *Staph. aureus* and PHA. Allele **2707* is significant for a lower response to the FMDV15 peptide for IgG1 and IgG2, and a low IFN-γ response. *DRB3*2707* is also nearing significance for the PBMC response to *Staph. aureus*, with an increase in PBMC proliferation. Allele **1601* is significant for a lower response to the FMDV15 peptide for IgG1 and IgG2. However, having the alleles results in a higher level of IgG to BHV-1 and is also associated with a lower response to PHA.

As observed in **Chapter 3,** amino acids within the pockets of the PBC were significantly associated with the humoral and cell mediated response to immunisation with a FMDV peptide. Here it is demonstrated that the amino acids within the PBC also have significant associations with the antibody response to whole virus vaccines and the cell mediated response to *Staph. aureus*. There are several positions within the PBC which are significant at different time points for the response to vaccination. However, no single position is significant across multiple vaccines or stimuli (Table 4.5 and 4.6). Interestingly several pocket positions were associated with the level of antibody prior to vaccination, with pocket 9 β37 being significant for BRSV IgG levels at day -28, but no significance was seen after vaccination. The specific antibody responses were not measured prior to vaccination for BHV1. However, at day 0 pockets 7 β67 and pocket 9 β57 were significant.

The centrally located pocket 4 has several amino acids which were significant. An asparagine at β74 (in pocket 4) has a highly significant association with BRSV-specific IgG at all time points post-vaccination, with animals possessing an asparagine having lower antibody levels. This position was also significant for the levels of specific IgG1 and IgG2 to FMDV15 (Table 4.5). Position β70 was significantly associated with the response to PIV3 and BRSV, and furthermore this position was also significantly associated with the IgG1, IgG2 and IFN-γ response to FMDV15 (Baxter et al. 2009). However, the presence of glutamic acid (E) or arginine (R) in this position had opposite effects in that E was associated with higher PIV3 antibody levels and R was associated with lower BRSV IgG, whereas R was associated with higher FMDV-specific IgG1, IgG2 and IFN-γ levels. In **Chapter 3** Figures 3.13 and 3.14 indicate that a change in amino acid at this position results in a conformational and electrostatic change in the PBC, which may therefore have an effect on the binding affinity of processed peptides.

There are 19 associations of vaccine response with pocket positions, with 7 being within pocket 7 (Table 4.5). Although this is less than for the response to FMDV it is important for any future vaccine design to consider the role of other pockets.

The pockets within the PBC have been associated with mastitis: Sharif et al (2003) found that a glutamine at position β74 was associated with the occurrence of mastitis. In this study no association was found between β74 and the PBMC proliferation to *Staph. aureus*, only pocket 4 position β71 was significantly associated. In addition certain motifs have been associated with either resistance or susceptibility to a range of mastitis-causing pathogens, with a Q-S-S-Y motif at positions β9-11-13-30 being associated with susceptibility and a Q-H-G-H motif at the same position being

associated with resistance. Whilst this chapter has not investigated the effect of motifs on the PBMC proliferation, only position β11 (pocket 6) was significant, but neither an H nor an S at this position was significant. It is interesting however to note that there are no significant pocket positions for PHA.

It would be unlikely to find single alleles which have a beneficial effect on the response to all pathogens, as due to the function of the MHC molecules, each allele would have positive or negative associations. Instead it would be more likely to find groups of alleles with similar motifs which may be associated with a wider range of epitopes. The pocket information provides a means of investigating whether there are any motifs or specific positions associated with different responses to a wide range of pathogens. The combined data in Tables 4.5 and 4.6 indicate that there are more associations with pocket positions than with alleles. From this analysis a few positions stand out as having more associations, particularly those within pocket 4 and 7/9, especially positions β70, 71 and 61. Position β70 within this population has a limited number of amino acids, E, R or Q. From the analysis, having a positive R at this position resulted in higher levels of IgG1 and IgG2 to FMDV15 and IgG to BRSV; however, there was a lower level of IgG to PIV3. This suggests that in terms of vaccine design, pocket 4 and 7/9 may have a larger effect and it would be interesting to further explore the associations with these pockets and other diseases and vaccines.

Investigating the associations of the *DRB3* alleles and pockets to the different antigens (either peptide, vaccine or bacteria) and comparing the different immune responses (humoral and cellular) has given a much clearer picture of the roles that

the polymorphisms within the Class II *DRB3* gene can have. It highlights the complexity of the immune response and provides avenues for further research.

Measuring the antibody response to vaccination may not reveal all the mechanisms involved in producing long lasting protection through vaccination. Instead it would be beneficial to understand the role of the cellular response. This information, in conjunction with knowledge of protective epitopes and the alleles of the BoLA genes, could be used to design more effective vaccination programs. Understanding the genetics underlying the response to vaccines could lead to increased efficacy through improvements in design. This would greatly improve animal welfare and reduce the cost of infectious disease in livestock. The identification of candidates for selective breeding for disease resistance would reduce the levels of intervention, such as culling and use of antibiotics.

5 Microarray typing of *BoLA-DQA* and Class I and qPCR for copy number of *BoLA-DQA*

5.1 Introduction

The BoLA region is highly complex with a high degree of polymorphism observed within the classical genes. In the BoLA Class II region there is a duplication of the polymorphic *DQ* genes in many haplotypes which further adds to the complexity of the region. This makes it difficult to accurately assess the polymorphisms in these genes and the possible role they play in response to pathogens and vaccines, thus a robust and accurate method of typing is required.

The bovine classical Class I genes are polymorphic and have been associated with mastitis (Aarestrup, Jensen, & Ostergard 1995) but due to poorly defined haplotypes, studying the exact polymorphisms which have an impact on immune response has proved difficult (Ellis et al. 2005).

The BoLA Class II region has duplicated *DQ* genes in about half of all the known haplotypes (Andersson & Rask 1988; Ballingall, Luyai, & Mckeever 1997) which has led to problems in typing these loci accurately. Moreover, the duplication of the *DQ* genes has been linked to immune responsiveness (Glass, Oliver, & Russell 2000; Park et al. 2004; Norimine & Brown 2005; Takeshima et al. 2008). It has been demonstrated that when the *DQ* genes are duplicated then both sets are expressed, thereby increasing the repertoire of T cell restriction (Xu, Park, & Lewin 1994).

Therefore the *DQA* genes are good candidates for the study of general disease resistance.

5.2 Microarray typing of BoLA genes

Studies investigating genes which are involved in response to pathogens or vaccination benefit from using large numbers of animals. To type large populations at specific loci a robust, cheap and high through-put genotyping technology is essential. Microarray technologies are relatively accessible and offer an inexpensive method of accurately typing large numbers of DNA samples quickly.

As the *DQ* and Class I genes are complex and have many polymorphisms, accurate typing has been problematic in the past (**Chapter 2**). A typing microarray for interrogating the alleles of some of the highly polymorphic genes would enable associations studies, e.g. on mastitis (Park et al. 2004), to be carried out in whole herds.

Microarrays have been primarily used in the detection of gene expression from RNA/cDNA. A typing array works in a similar fashion, with probes being 'spotted' onto glass slides followed by the hybridisation of 'test' DNA (Call, Chandler, & Brockman 2001). DNA microarrays have been used to study microbial genomics (reviewed by Cummings & Relman (2000)) and host genomics (reviewed by Jares (2006)). The fluorescent pattern of the hybridised probes allows for the identification of alleles. This then enables many DNA samples to be simultaneously typed at a specific locus.

5.3 *DQA* duplication

For many years Southern blots and *in situ* hybridisation have been used to test for copy number variations in genes. However, these techniques are limited as they can be time-consuming, laborious and require large quantities of DNA (Hoebeeck, Speleman, & Vandesompele 2007). An alternative method is to utilise real-time quantitative PCR techniques, which are reliable, fast and can be used on limited quantities of DNA. Real-time qPCR offers the possibility of quickly and reliably detecting *DQ* gene duplication.

5.4 Aim

This chapter aims to investigate microarray typing for the *BoLA DQA* genes and the MHC Class I region. In addition this chapter aims to investigate the feasibility of using real-time qPCR for assessing the copy number of *DQA* genes.

The microarray technique which was designed by Dr. Chris Davies (Park et al. 2004) presented a great opportunity to type the RoBoGen animals at the MHC Class I loci. To do this I raised suitable funds for a 6 week visit to Dr Chris Davies' laboratory at Washington State University, U.S.A. This provided an opportunity to learn a novel technique which could then be implemented at The Roslin Institute.

.

5.5 Materials and methods

5.5.1 Microarrays

5.5.1.1 Samples

Genomic DNA from the RoBoGen herd was used for all the microarray experiments (**Chapter 2,** sections 2.3.1 and 2.3.2).

5.5.1.2 PCR protocols for microarray

All PCR amplification protocols and oligonucleotide primers were designed by Dr. Chris Davies (presently at Utah State University), to amplify all known MHC Class I alleles at exon 2 and exon 3 and *DQA1* and *DQA2* alleles (primer sequences Appendix D.1, D4 and D.6).

5.5.1.3 MHC Class I exon 2 PCR

PCR was carried out on ~200 ng genomic DNA. The PCR reaction mix was: 1 unit Taq polymerase (Fisher Scientific, USA), PCR 10X buffer B (Fisher Scientific), 25 mM $MgCl_2$ (Fisher Scientific), 25 mM dNTPs (Invitrogen, USA) and diethylpyrocarbonate (DEPC)-treated water (Invitrogen). Primers were 0.8 µM biotinylated forward primer BoC1FP-E2A (5'd[biotin-ACG TGG ACG ACA CGC ACT TC] 3') and 0.8 µM biotinylated reverse primer BoC1RP-E2A (5'd[biotin-CTC GCT CTG GT GTA GTA GCC]3') in final volume of 50 µl. Briefly the thermal set-up was denature at 94^{o}C for 4 min, followed by 35 cycles of 94^{o}C for 1 min, 54^{o}C for 30 sec and 72^{o}C for 1 min and 30 sec followed by a final extension for 5 min at

72°C. Samples were run on a 1.5% agarose gel. PCR products were stored at -20°C until use.

5.5.1.4 MHC Class I exon 3 PCR

The PCR conditions for the amplification of exon 3 are the same as section 5.5.1.3 but using primers; BoC1FP-E3D (5'd[biotin-TGG TCG GGG CGG GTC AGG GTC TCA C]3') and BoC1RP-E3C (5'd[biotin-CCT TCC CGT TCT CCA GGT ATC TGC GGA GC]3'). The annealing temperature was 64°C.

5.5.1.5 DQA exon 2

The PCR conditions for DQA are the same as the previous protocol (section 5.5.1.3) but with the following primers; 0.8 µM BoDQA1FP-E2A 5'd[biotin-CTCCGACTCAGCTGACCACATTGG]3'), 0.8 µM Biotinylated Forward Primer BoDQA2FP-E2B (5'd[biotin-CCTCAATTATCAGCTGACCACGTTGG]3'), 0.8µM Biotinylated Reverse Primer BoDQA1RP-E2A (5'd[biotin-TACTGTTGGTAGCAGCAGTAGAGTTGG]3') and 0.8 µM Biotinylated Reverse Primer BoDQA2RP-E2B (5'd[biotin-GGTGGACACTTACCATTGATAACAGGG]3'). The annealing temperature was 58°C.

5.5.1.6 Class I exon 2 and exon 3 microarrays

Class I exon 2 and exon 3 microarrays were printed at Washington State University, USA by Dr Chris Davies (Call, Chandler, & Brockman 2001; Park et al. 2004; Davies et al. 2006). Briefly the probes (Appendix D.5 and D.7) were printed on Teflon masked microscope slides (Tekdon's Masked slides, Florida) using a

microgrid II arrayer (Genomic Solutions, Ann Arbor, MI, USA). In total 104 oligonucleotides (20-22 bp) probes were printed in duplicate on the slides for both exon 2 and exon 3. The probes were designed using an alignment of all known Class I sequences.

5.5.1.7 DQA microarray

The DQA microarrays were printed at Utah State University (Logan, USA) by Dr. Chris Davies. The arrays were printed on Teflon masked glass slides (Tekdon's Masked slides, Florida) using a microgrid II arrayer. In total 58 oligonucleotides (20-22 bp) probes were printed in duplicate on to the slides (Appendix D.2). The probes were designed using an alignment of all the DQA alleles defined to date (http://www.ebi.ac.uk/ipd/mhc/bola/).

5.5.1.8 Hybridising PCR products to array

The slides were washed twice with double distilled (dd) H_2O at 50^{o}C for 5 minutes on a plate shaker (~45 rpm) followed by a wash in ddH_2O at room temperature (RT) for 5 min on a plate shaker (~45 rpm). The slides were dried using a minifuge (ArrayIT, USA) for 10-15 sec and were then placed into a hybridising chamber with 5X SSPE (3 M NaCl, 0.2 M NaH_2PO_4-H_2O, 0.02 M EDTA dissolved in H_2O). 40 µl 1X pre-hybridisation (50X Denhardt's (Invitogen), 20X SSPE) was placed on each well in the slide. The hybridisation chamber was sealed and left at RT for 30 min.

For each PCR sample 12 µl of product was mixed with 79 µl 1.14X hybridisation (50X Denhardt's, 20X SSPE) solution. The PCR product/hybridisation solution was then denatured at 95^{o}C for 5 min in a heat block and cooled rapidly on ice. Using a

pipette, the pre-hybridisation solution was removed from each of the wells and was replaced with 40 µl of PCR product/hybridisation solution. Each sample was hybridised in duplicate (2 separate wells). The hybridisation chamber was closed and placed in a sealed plastic bag to incubate overnight at 50°C.

The slides were then removed from the chamber and washed twice in 2X SSPE with 10% SDS at RT for 5 min followed by two washes in 0.5X SSPE at RT for 5 min each. For 5 slides a conjugation solution was prepared with 36 ml H_2O, 10 ml 20X SSPE, 4 ml 50X Denhardt's (Invitrogen) and 20 µl Streptavidin-Alexa Fluor 555 conjugate (Invitrogen). 50 ml of conjugate solution were added to each slide staining jar and left in the dark at RT for 30 min on a plate shaker. After 30 min each slide was washed twice in 0.5X SSPE at RT for 5 min and spun dry in a minifuge for 10-15 sec. Slides were then stored in the dark until they were scanned.

5.5.2 Scanning of Slides

5.5.2.1 Class I exon 2 and exon 3 slides

The Class I exon 2 and exon 3 slides were scanned at Washington State University using an ArrayWorx (USA) scanner and all images were saved as .jpeg files.

5.5.2.2 DQA exon 2 slides

The slides were scanned using the Ark Genomics facility (http://www.ark-genomics.org) on an Axon GenePix scanner at a wavelength of 532 nm (Molecular Devices, USA) and images were saved as .jpeg files.

5.5.2.3 Editing of scanned images

The jpeg files were edited using Adobe Photoshop (Adobe, USA). The images were auto-corrected using auto-levels and auto-contrast. The duplicated wells were placed side-by-side into a new document to allow easier analysis of the hybridisation patterns.

5.5.2.4 Analysis of Class I array microarray

The edited Photoshop images were scored subjectively by eye according to the hybridisation scale (Figure 5.1). Each sample was hybridised in duplicate (2 wells) and each spot was printed in duplicate in each array. The scoring of the spots used all four replicates and a subjective average score was determined. Each score was entered into a simple program, CYTOFILE matrix analysis software (Davies et al. 1994), which re-arranges data into a new file. The program first organises the data into groups dependent on probe groupings (Appendix D.8), then arranges into similarity. When all of the allele specific probes scored at least 6, the sample was scored as a positive for that allele.

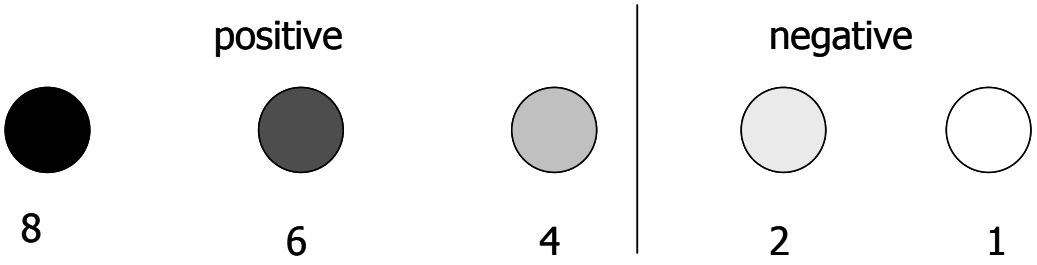


Figure 5.1 Rough scale of hybridisation for the Class I and DQA typing microarrays, for subjectively scoring of probe hybridisation on the microarrays.

5.5.2.5 Analysis of DQA microarray

As the DQA microarrays were hybridised at The Roslin Institute, a new method of data analysis was investigated. The slides were scanned on an Axon scanner (Molecular Devices, USA) using a 10 micron scan and GenePix Pro software (Molecular Devices) was utilised for the analysis. Each probe was identified using a .gal file template to allow quantification. The measurement of intensity of each spot was then transferred into a Microsoft Excel (Microsoft, USA) spreadsheet format. The spreadsheet contained information on the position of the spot, probe name (Appendix D.1, D.2 and D.3), size of spot and intensity of fluorescence.

5.5.2.6 Assignment of haplotypes and alleles

The results from the CYTOFILE program (described in section 5.5.4.2) allow the allocation of Class I haplotypes and *DQA* alleles. The probes were designed based on the polymorphic regions in the known Class I and *DQA* sequences by Dr Chris Davies. The grouping together of the probes is based on the full Class I and *DQA* sequences, for example an animal positive for the A14 haplotype would have positive probes from the N*02301 and N*02401 sequences.

5.5.3 qPCR *DQA* genes

As duplication in the *DQA* and *DQB* genes is likely to have an effect on the immune response, it would be useful to have a rapid and easy way of identifying animals with the duplication. A qPCR method offers a way of assessing copy number in the genome and was considered to be a possible avenue for use with the *DQA* genes.

5.5.3.1 DNA samples

The genomic DNA used in the qPCR assay was extracted from bovine cell lines (Table 5.1) using a Qiagen DNeasy blood and tissue kit (Qiagen, UK), following the manufacturers instructions. The cell lines were chosen as they had previously been typed at the *DQA* locus (Davies et al. 1994; Glass, Oliver, & Russell 2000) or had an inferred number of *DQA* genes based on the *DRB3* alleles and therefore have a known copy number of *DQA* genes to allow validation of this technique.

Cell line ID numbers and typing	Workshop ID	Workshop DH Haplotype	*DRB3* alleles	Number of *DQA* genes#
10814 (a) and (b)	WK-50	15B/24A	*1001/*0101	2
10795 (a) and (b)	WK-029	08A/24A	*1201/*0101	3
10775 (c)	ND	03A/24A	*1001/*0101	3
10757(c)	ND	24A/28A	*0101/*0701	3
2390 (c)	ND	18A/18A	*1801/*1801	4
2858 (b)	WK-31	03A/22C	*1001/*1101	4
2859 (b)	WK032	03A/18A	*1001/*1801	4
10812 (b)	WK-53	18A/28A	*1801/*0701	4

(a) Glass, Oliver, & Russell 2000 (b) 5th BoLA Workshop (Davies et al. 1994) and (c) Glass unpublished data.
 # Except in the case of cell line 10814 and 10795, the number of DQ genes was inferred from the typing of the *DRB3* alleles and available DR-DQ haplotypes Appendix D.9.
ND Not done at workshop.

Table 5.1 Cell line DNA used in DQA copy number qPCR assay and inferred DQA copy number (alleles).

5.5.3.2 Designing and optimisation of primers

Exon 2 of DQA is highly polymorphic and it was not possible to design suitable primers, so, using an alignment of DQA sequences, primers were designed for the exon 3 region. Primers DQAex3F (5'[CCC AAC ACY CTC ATC TGT CA]3') and DQAex3R (5'[CAG TGC TCC ACT TTG CAC TG]3') were designed using DQA*0101 as a template in Primer3 (Rozen & Skaletsky 2000). A degenerate base was used at position 9 to enable amplification of all known alleles. The amplification protocol was as follows: 10X buffer (Bioline), $MgCl_2$ at two concentrations (1.5 mM or 2.0 mM), DQAex3F 15 pmol, DQAex3R 15 pmol, 25 mM dNTPs (Bioline), H_2O

and 1 unit Taq polymerase (Bioline). Cycles were run at 3 different annealing temperatures 55°C, 56°C and 57°C. The PCR cycle consisted of 95°C for 10 min followed by 30 cycles of 95°C for 1 min (annealing temperature; 55°C, 56°C or 57°C) for 1 min and 72°C for 1 min, followed by a final extension of 72°C for 5 min.

5.5.3.3 Sequencing

The PCR products from the DQA touchdown protocol with 10% DMSO were sequenced using the same method as described in **Chapter 2,** section 2.3.4. From the sequencing it was possible to see that there was an error in the design of the forward primer of DQAex3F, as the consensus obtained from http://www.ebi.ac.uk/ipd/mhc/bola/ had a G at 242 bp. However, it appeared that at this position the base is either polymorphic or is a C, but from 8 sequenced animals this base was consistently a 'C'. The primer was re-designed using this information to DQAex3Fv.2 (5'[CAC TGC AAA GTG GAG CAC TG]3') (Figure 5.2). A new annealing temperature of 55°C was used with 10% DMSO and 5 pmol primer and the resulting product was run on a 1.5% agarose gel.

5.5.4 qPCR assay

5.5.4.1 Design of Taqman probe for DQA

A non-extending oligonucleotide probe was designed using Primer3 software to anneal to the DQA exon 3 sequence between the two amplification primers (DQAex3Fv.2 and DQAex3R), using the consensus sequence information from http://www.ebi.ac.uk/ipd/mhc/bola/. The DQA Taqman oligonucleotide probe sequence is (5'[AYG GGC AYK YRG TCA YAG AG]3') (Figure 5.2). The

Taqman® MGB (Applied Biosystems, USA) probe is in the middle of exon 3 and was labelled with 6FAM (6-carboxyflourescein) fluorescent dye at the 5' end.

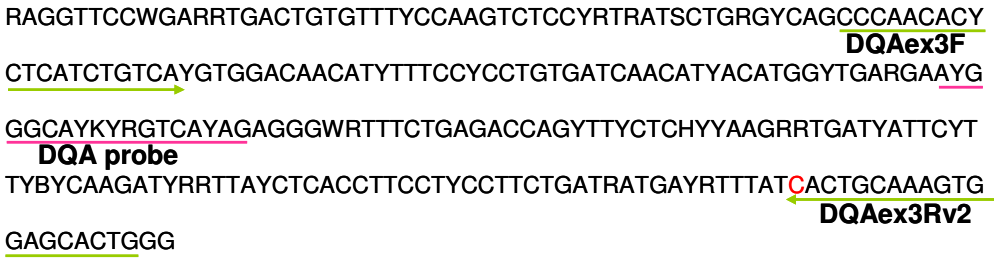


Figure 5.2 DQA exon 3 consensus sequence. qPCR primer positions are underlined in green and probe position is underlined in pink. The red 'C' in the reverse primer is the base changed from the original design after sequencing.

5.5.4.2 *DRA* control gene

The *DRA* gene was chosen as a control for the qPCR assay as it encodes the alpha chain of the DR molecule and so was considered to be similar to the DQA gene. DRA is monomorphic and only a single copy is present in the bovine genome. DRA primers DRAex3F (5'[TG ACTT TGA TGG TGA TGA GA] 3') and DRAex3R (5' [GAG CGC TTT ATC ATG ATC TC] 3') and non-extending oligonucleotide probe (5' [AAT TTG GAC ATT TTG CCA GA]3'), which was labelled with VIC, were designed with Primer3 software. The PCR for the DRA gene used the same conditions as the PCR for the DQA gene.

5.5.4.3 Quantitative real-time PCR conditions

The final qPCR protocol was used for both DQA and DRA primers and probes. Briefly the reaction contained: 2X Taqman buffer (Applied Biosystems, USA), 5 pmol DQAex3Fv2, 5 pmol DQAex3R, 10% DMSO, 250 nM Taqman® MGB probe

DQA (Applied Biosystems), and H_20 in a final volume of 20 µl. The PCR was run using the same conditions as stated previously (section 5.5.3.2). The qPCR was run on a Stratagene (USA) qPCR machine and analyzed with Mx3000 and MxPro (Stratagene, USA). All samples were carried out in triplicate.

5.6 Results

5.6.1 MHC Class I

A total of 108 samples from the RoBoGen herd, which had been typed at the *DRB3* locus using the SBT technique (**Chapter 2**) and represented all of the *DRB3* alleles in this herd, were hybridised to the MHC Class I exon 2 and exon 3 microarray slides. The hybridisation of the PCR products allowed clear identification of positive spots (Figure 5.3). The spots were scored using the subjective scoring method described previously (Figure 5.1). From Figure 5.3 it is possible to easily identify the strongly positive spots e.g. the G1, H1 pair and also those which are still positive but not as strong, e.g. the A2, B2 pair. Using the hybridisation pattern of both exon 2 and exon 3 arrays, 82 animals were successfully allocated MHC Class I haplotypes using the CYTOFILE program example of output shown in Figure 5.4. The Class I probes were designed by Dr. Chris Davies, they included all known sequence data and serological defined haplotypes. The allocation of the haplotypes using the microarray identifies allele specific probes and the haplotypes to which the probes belong e.g. probes for N*01301 and N*01302 would all hybridise for haplotype A18. As Class I alleles have not been allocated to loci the Class I region the microarray uses the originally defined serology haplotypes (Davies et al. 1994) (Appendix D.8). However, due to the complexity of the Class I region, it was not possible to reliably

allocate haplotypes to 25 of the animals and a few of the animals' Class I haplotypes were ambiguous.

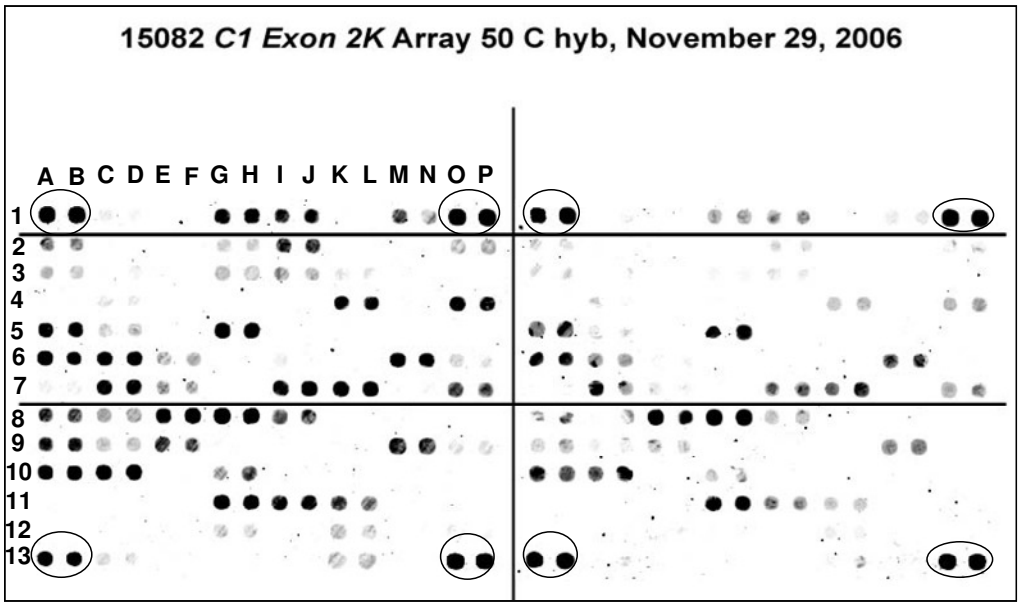


Figure 5.3 Microarray scanned and edited image of RoBoGen animal 15082 on Class I slide for exon 2. Each spot is printed in duplicate side by side, e.g. A1 and B1. The circled spots are positive controls (A1, B1, O1, P1, A13 B13 O13, P13). The image has grid lines to allow easier identification of probes. The image represents a typical example of a hybridised microarray.

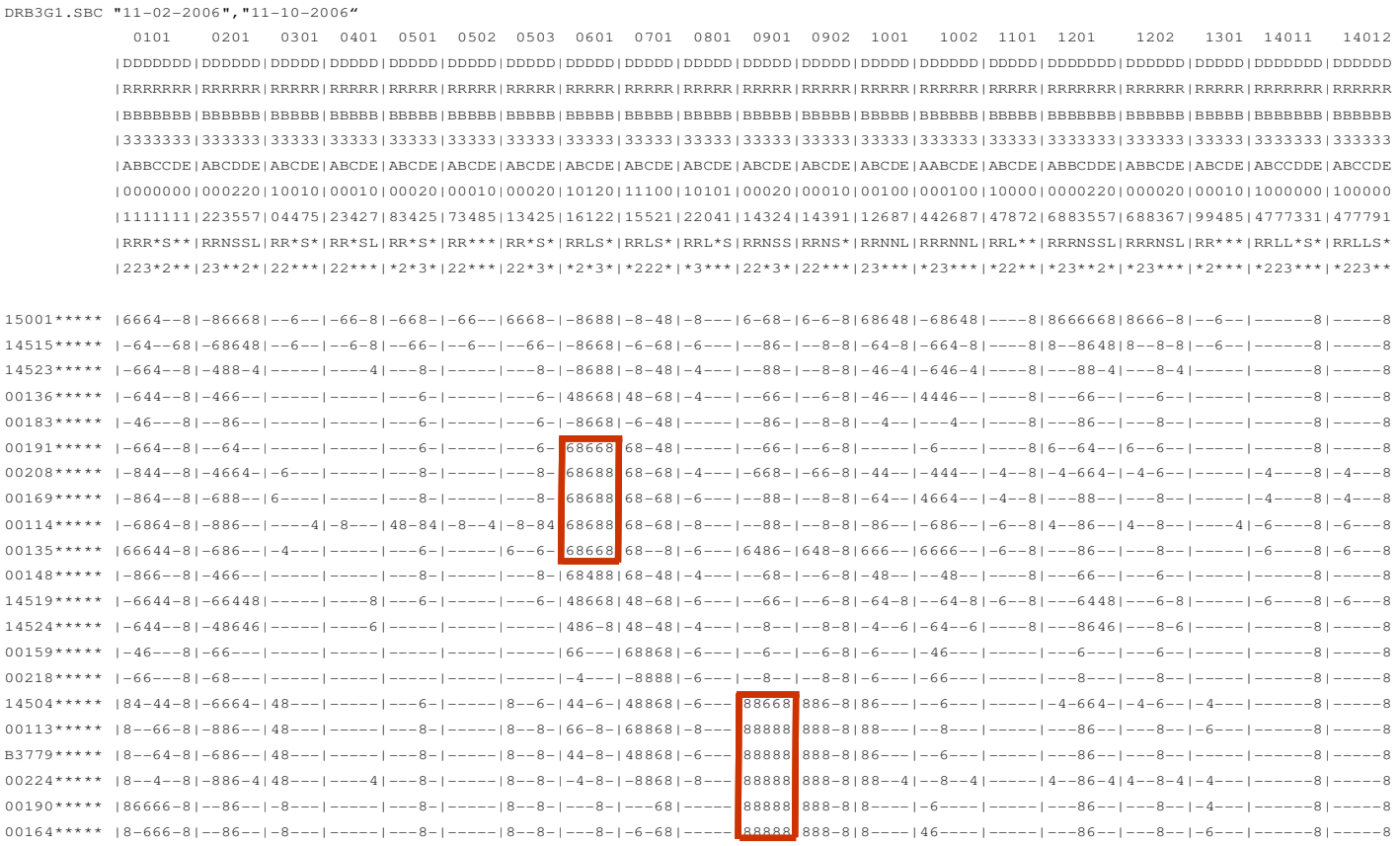


Figure 5.4 An example of data output from CYTOFILE. Red boxes indicate positive probes grouped together to allow easier identification of alleles/haplotypes. Alleles across the top with probes underneath (read from top to bottom), animal identification numbers listed on left hand side.

The most common haplotype seen was A12 which was found in 54% of the animals tested on the microarray (Table 5.2). The least common haplotypes were A70, A71 and A72 (definition of haplotypes by Dr C Davies, personal communication). A single animal, animal 164, had four MHC Class I haplotypes defined by the microarray, A33, A06, A18 and A61.

It was possible to hypothesise Class I-DRB3 haplotypes from the results of the Class I microarrays and the *DRB3* typing (Table 5.3). The most convincing haplotype was for *DRB3*2707* and Class I, A12 due to the large number of animals with this haplotype (n=34).

Haplotypes found in RoBoGen herd	Allele assigned to haplotypes#	Number of animals (from RoBoGen selection)
A06	N*01401 N*01402 N*01501 N*01601	7
A18	N*01301 N*01302	7
A12	N*01901 N*02001 N*00801	44
A10	N*00101 N*00102 N*00103 N*00201 N*01101 N*01201	3
A13	N*03101 N*03201N	5
A14	N*02301 N*02401	25
A15	N*00901 N*02401 N*02501	5
A19	N*01401 N*01402 N*01601	7
A20	N*02601 N*02602 N*02701 N*02702	3

A31	N*02101 N*02201	2
A33	N*00401 N*00501	9
A67*		11
A68*		1
A70*		1
A71*		1
A72*		1

Table 5.2 MHC Class I haplotypes defined by microarray and alleles (# http://www.ebi.ac.uk/cgi-bin/ipd/mhc/view_nomenclature) and number of animals. (* haplotypes defined by Dr Chris Davies, personal communication).

***DRB3* allele**	**Class I haplotypes**	**No. of animals**
**2707*	A12	34
**1101*	A14/A67	6
**1601*	A33	7
**0201*	A14	6

Table 5.3 Hypothesised DRB3/Class I haplotypes from the microarray and SBT results in the RoBoGen herd.

5.6.2 DQA

The PCR for DQA produced a clear product which was then hybridised to the microarray slide. The slides were scanned using an Axon scanner and produced an image of the whole slide (Figure 5.5). Initially an attempt was made to use the GenePix software to identify spots and to measure their intensity. The results from the Genepix software indicated difficulties in locating the positive spots, as they varied in position and size between the different arrays. The Excel spreadsheet of the probe intensities showed a huge range of scores for positive hybridisations, for example a control probe

had an average intensity of 6933, while a positive test probe had an intensity of 11887 which is nearly double the value. In addition it was found that the values for the positive spots varied between the arrays, with a control probe having an intensity score of 19358 on 1 array and the same probe having a score of 8863 on another array on the same slide. This variation in intensities meant that it was impossible to automate the scoring of probes.

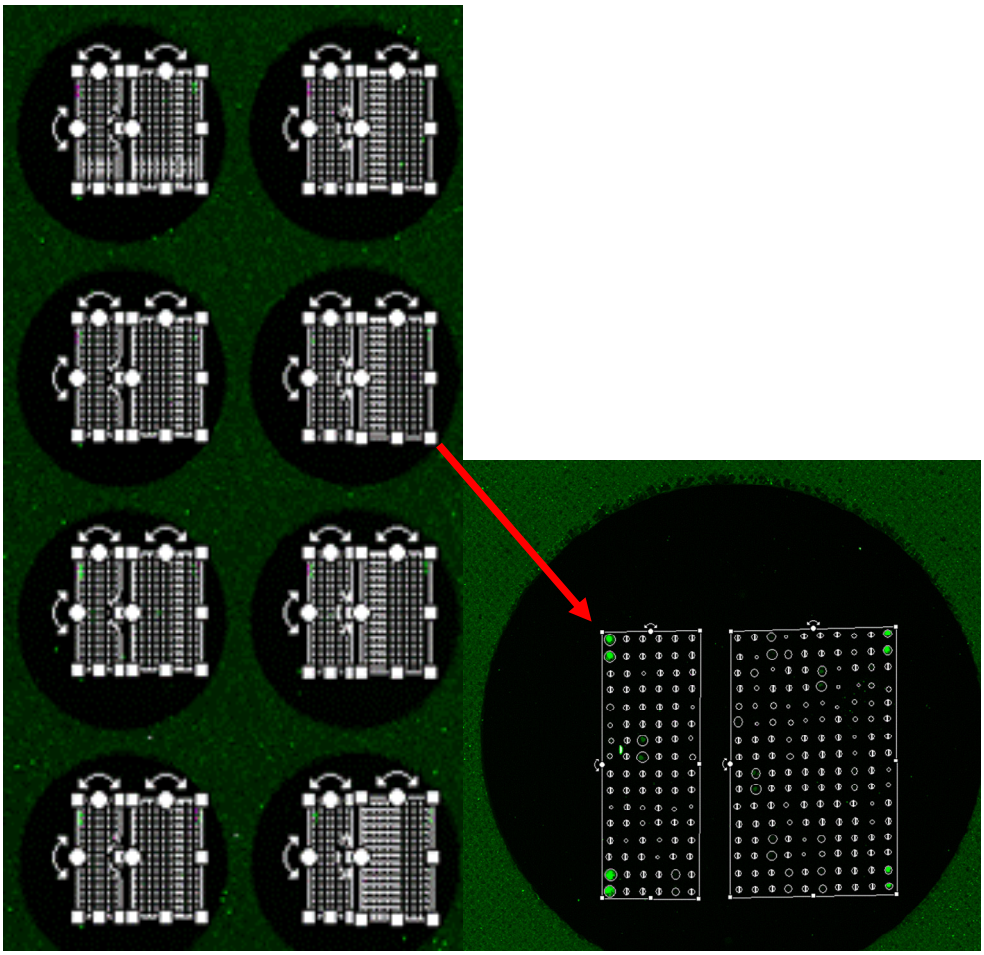


Figure 5.5 Image from GenePix software of entire microscope slide with 8 microarrays, with .gal file template overlaid on image. The magnified picture shows 1 microarray (1 sample). Each of the green positive spots has been identified by the GenePix .gal file.

The scoring of the DQA slides was done manually using the Photoshop .jpeg images. However, the majority of the scanned images were poor in quality and this is highlighted in Figure 5.6. Using Photoshop it was possible to manipulate the images to allow the scoring of spots, although the accuracy of the scoring is reduced (Figure 5.7). The images of the slides were sent to Dr. Chris Davies (Utah State University) and he allocated alleles, based on his previous microarray knowledge.

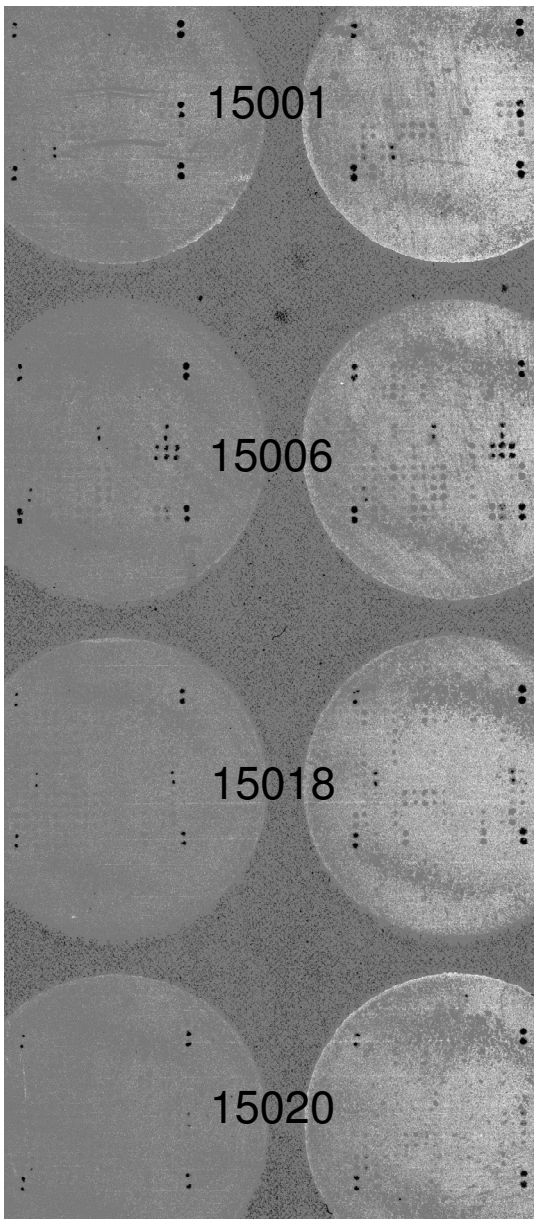


Figure 5.6 Image of scanned slide of DQA microarray showing entire scan area. Animal identification numbers in black and duplicates side by side. This image illustrates the poor quality of the scanned images.

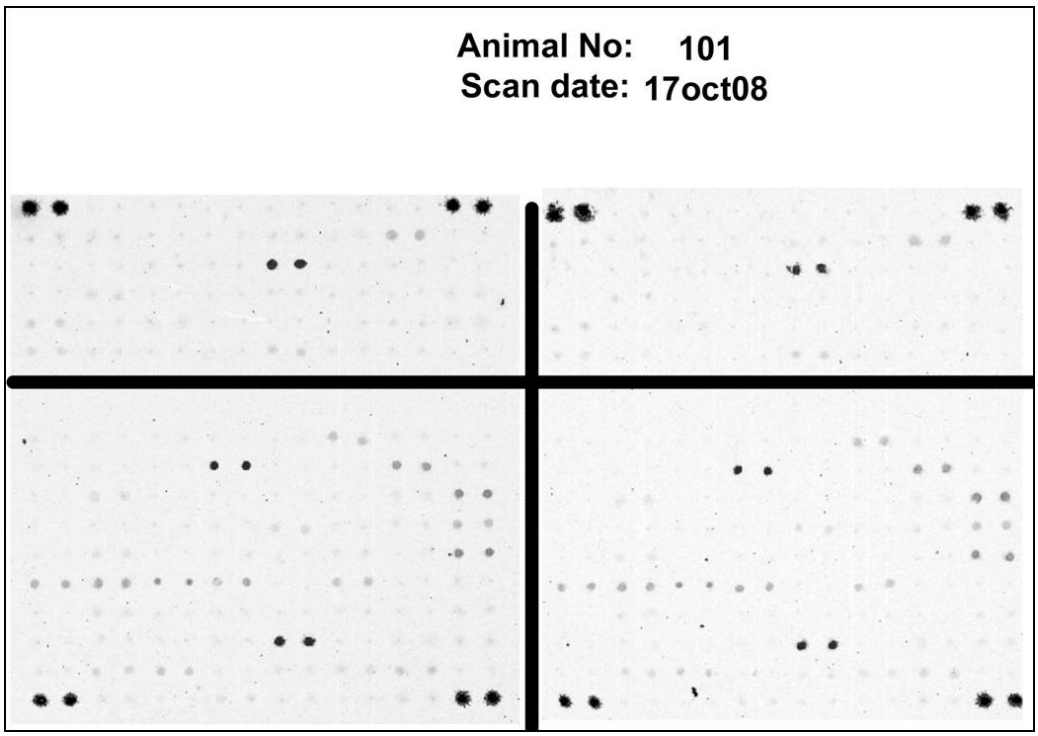


Figure 5.7 Microarray scanned and edited image of RoBoGen animal 101 on DQA array showing both replicates. Each spot is printed in duplicate side by side. The image has grid lines to allow easier identification of probes. The image represents a typical example of a DQA hybridised microarray.

Due to the difficulty with haplotype allocation discussed in section 5.6.2, only 31 complete DQA alleles/haplotypes were allocated for the 78 animals used in the microarray experiment. There were 7 animals which had partial identification of haplotypes with a single *DQA* allele. Eighteen *DQA* alleles were identified in 38 animals (Table 5.4). Fifteen of the alleles were in duplicated haplotypes. The most common duplicated haplotype in the herd was *DQA2*12021*, *DQA2*22021* which was found for 6 animals, all of which had *DRB3*1601*. The *DRB3*2707* allele, which is the most common *DRB3* allele, was found with 2 different *DQA* haplotypes, either *DQA1*0203* or *DQA1*1203* and *DQA2*2201*.

DQA allele	No. of animals for each for allele	duplicated
*DQA1*0101*	3	yes
*DQA1*0102*	1	no
*DQA1*0203*	7	no
*DQA1*0302*	6	no
*DQA1*10011*	2	yes
*DQA1*10012*	5	yes
*DQA1*12011*	3	yes
*DQA1*12021*	3	yes
*DQA1*1203*	5	yes
*DQA2*2101*	6	yes
*DQA2*2201*	12	yes
*DQA2*2202*	1	yes
*DQA2*22021*	8	yes
*DQA2*22031*	1	yes
*DQA2*2206*	1	yes
*DQA3*25012*	4	yes
*DQA3*25013*	1	yes
*DQA3*2901*	1	yes

Table 5.4 DQA alleles identified with the typing microarray in 38 RoBoGen animals and their duplication status.

5.6.3 qPCR

DQA and DRA primers were successfully designed to amplify exon 3 from the monomorphic DRA and the polymorphic and duplicated DQA genes. TaqMan® non-extending MGB^{TM} nucleotide probes were designed and used to measure the PCR amplification.

5.6.3.1 Touchdown qPCR

The samples from the touchdown qPCR (detailed in section 5.5.3.2) were run on a 1.5% agarose gel (Figure. 5.8).

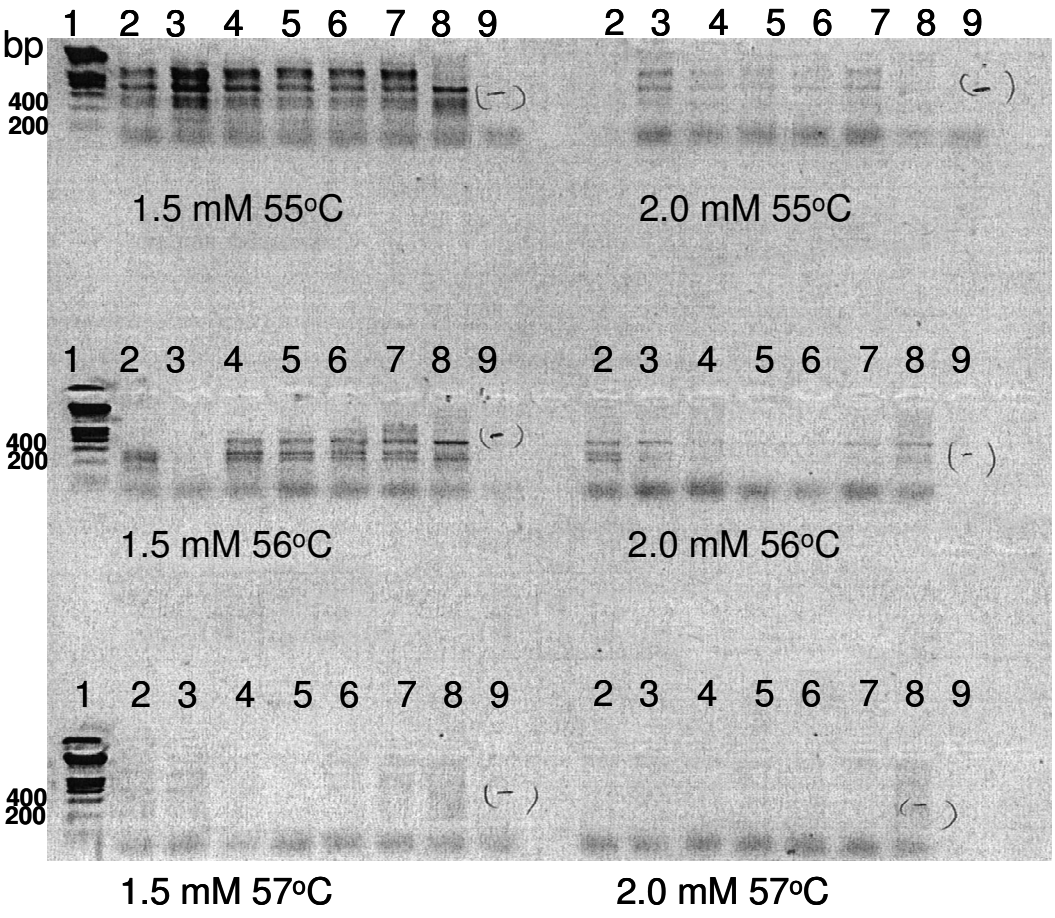


Figure 5.8 Agarose gel electrophoresis of DQA PCR amplification with primers DQAex3F and DQAex3R. Lane 1: hyperladder I (Appendix B.2), lane 2: Holstein cell line DNA animal 18014, lane 3: Holstein cell line DNA animal 18014, lane 4: Holstein cell line DNA animal 10757, lane 5: Holstein cell line DNA animal 10757, lane 6: Holstein cell line DNA animal 10795, lane 7: Holstein cell line DNA animal 10757, lane 8: RoBoGen DNA animal 101 and lane 9 –ve control. In –ve control lanes the primer dimer band is visible.

Multiple bands were seen for each animal, suggesting non-specific amplification of the DQA exon 3. A touchdown protocol was implemented to resolve this problem, with a starting annealing temperature of 57^{o}C decreasing to 54^{o}C by 1^{o}C every 2nd cycle. All other conditions were kept the same as in previous reactions with the addition of 5% DMSO (dimethyl sulfoxide) (Figure. 5.9)

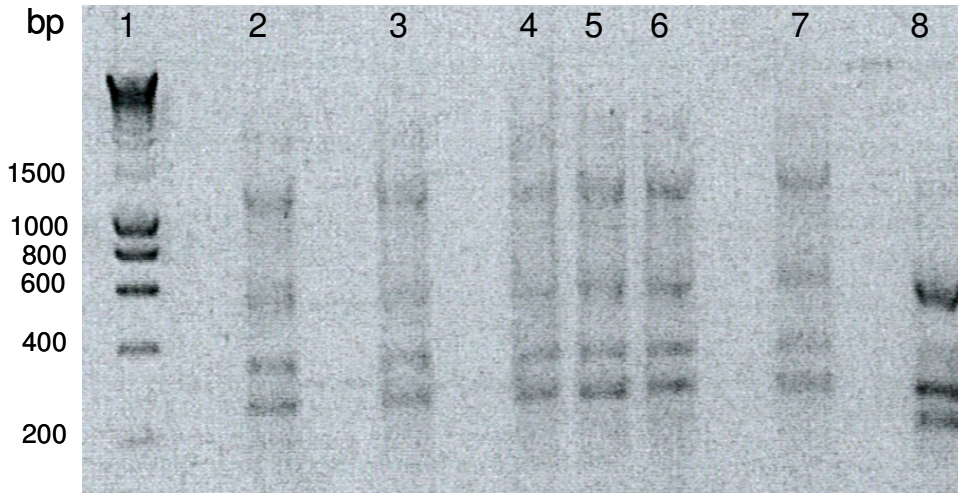


Figure 5.9 Agarose gel electrophoresis of DQA PCR amplification with primers DQAex3F and DQAex3R using 5% DMSO. Lane 1: hyperladder I (Appendix B.2), lane 2: Holstein cell line DNA animal 18014, lane 3: Holstein cell line DNA animal 18014, lane 4: Holstein cell line DNA animal 10757, lane 5: Holstein cell line DNA animal 10757, lane 6: Holstein cell line DNA animal 10795, lane 7: Holstein cell line DNA animal 10757 and lane 8: RoBoGen DNA animal 101.

From the touchdown PCR there were still too many non-specific bands of PCR product. Touchdown PCR was repeated using 10% DMSO and 5 pmol of each primer and run on a 1.5% agarose gel (Figure 5.10). The touchdown PCR showed a single PCR product

band (and primer dimer), which was purified for sequencing.

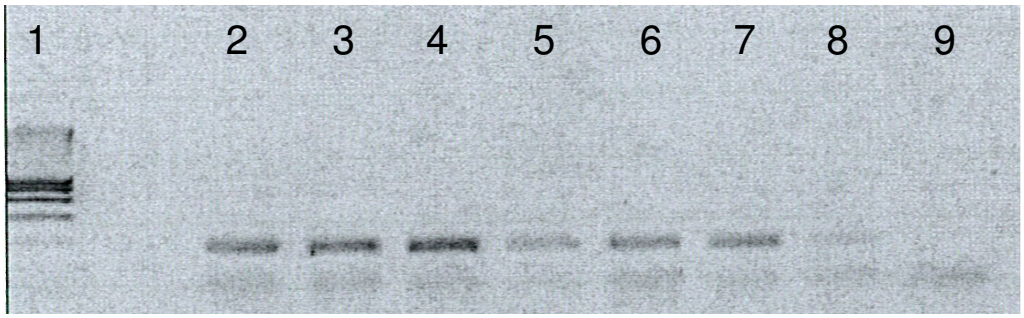


Figure 5.10 Agarose gel electrophoresis of DQA PCR amplification with primers DQAex3F and DQAex3R using 10% DMSO and touchdown PCR protocol. Lane 1: hyperladder I (Appendix B.2), lane 2: Holstein cell line DNA animal 18014, lane 3: Holstein cell line DNA animal 18014, lane 4: Holstein cell line DNA animal 10757, lane 5: Holstein cell line DNA animal 10757, lane 6: Holstein cell line DNA animal 10795, lane 7: Holstein cell line DNA animal 10757, lane 8: RoBoGen DNA animal 101 and lane 9 –ve control. Primer dimers are visible in all lanes.

5.6.3.2 Sequencing

The PCR products from the DQA touchdown protocol with 10% DMSO Figure 5.10 were sequenced using the same method described in **Chapter 2,** section 2.3.4. (an example of DQA exon 3 sequence shown in Figure 5.11). The sequence trace also confirmed that it was DQA exon 3 which was being amplified by PCR.

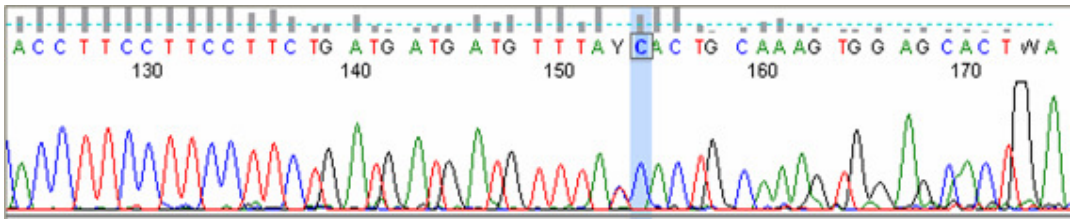


Figure 5.11 Fragment of sequence trace file of RoBoGen animal 101. The blue highlighted 'C' base lies in the forward primer DQAex3F.

The DRA and DQA genes were successfully amplified using the qPCR protocol (Figure 5.12). The amplification plots were obtained using the MxPro software (Applied Biosystems) (Figure 5.13). The Ct value at which the amplification crossed the

threshold was obtained from the amplification plots and used in subsequent calculations. The threshold value was kept the same for all samples.

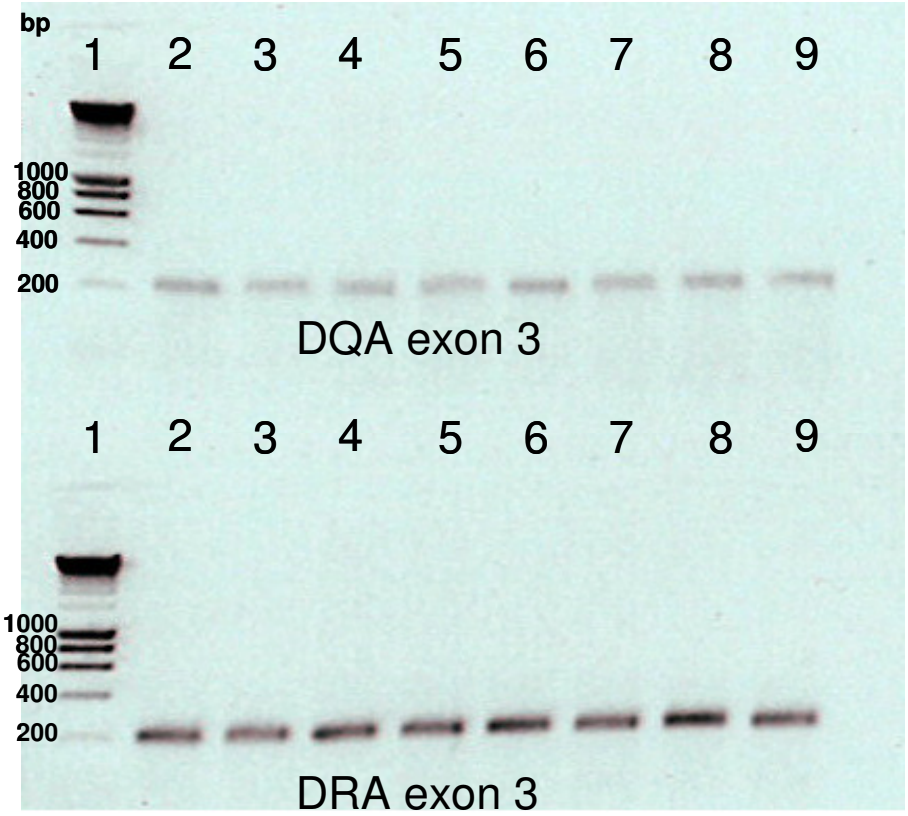


Figure 5.12 1.5% agarose gel electrophoresis of DQA and DRA PCR amplification with primers DQAex3F and DQAex3R (top row) and DRAex3F and DRAex3R (bottom row) Lane 1: hyperladder I (Appendix B.2); lane 2: Holstein cell line DNA animal 18014; lane 3: Holstein cell line DNA animal 18014; lane 4: Holstein cell line DNA animal 10757; lane 5: Holstein cell line DNA animal 10757; lane 6: Holstein cell line DNA animal 10795; lane 7: Holstein cell line DNA animal 10757; lane 8: Holstein cell line DNA animal 2858; lane 9: Holstein cell line DNA animal 2859.

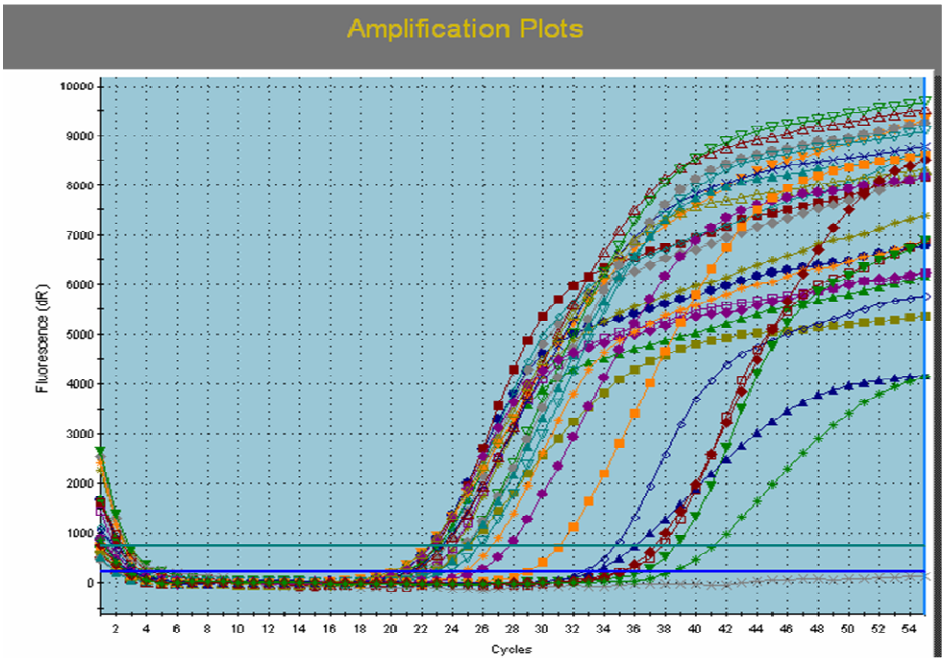


Figure 5.13 Screen shot taken from MxPro output. Each coloured line represents the amplification profile of a single DNA sample. This output shows the fluorescence (dR) of each sample and the number of cycles of amplification. The green horizontal line is the threshold measurement for JOE and the blue horizontal line the threshold measurement for FAM.

5.6.3.3 Analysis of qPCR

For the determination of copy number of the DQA gene the $2^{-\Delta\Delta Ct}$ method was chosen (Livak & Schmittgen 2001). To allow valid calculations using the $2^{-\Delta\Delta Ct}$ method the amplification efficiencies of both the DQA and DRA genes must be similar. The C_t values were measured over a tenfold dilution range of a control DNA that contains two copies of DQA and DRA (cell line animal 10814 (Glass et al. 1991)). Standard curves were created and an R^2 value obtained, (R^2 was 98% for both the DQA and DRA). The determination of the real-time qPCR efficiencies of the reference gene, DRA, and the target gene, DQA, were calculated according to the equation $E=10^{(-1/slope)}$ (Pfaffl 2001). The efficiencies were similar for the DQA and DRA gene PCR amplification (E=2.25 and E=2.31 respectively) (Figure 5.14) allowing the valid use of the $2^{-\Delta\Delta Ct}$ method.

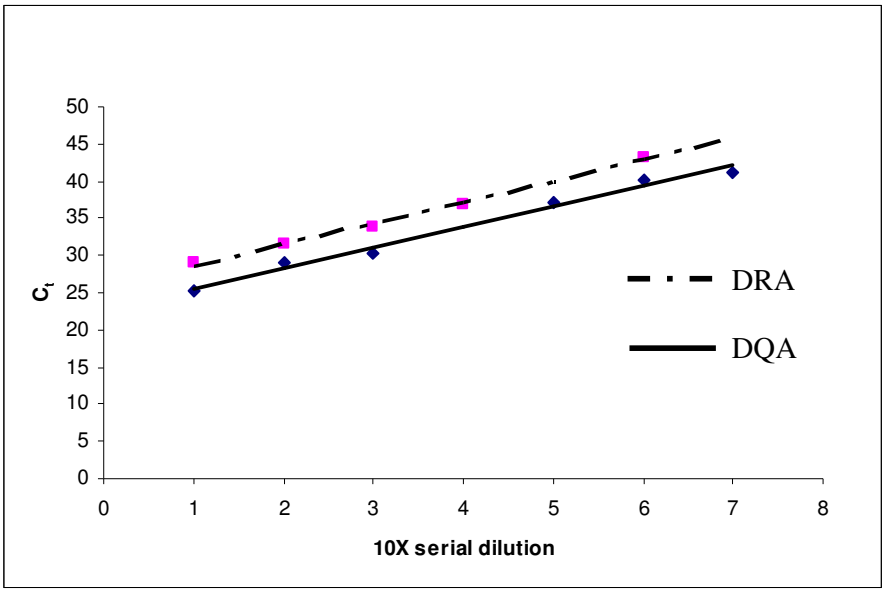


Figure 5.14 Determination and comparison of the PCR efficiency for DQA (Efficiency 2.31) and DRA (Efficiency 2.31) genes.

The gene copy number was calculated by the formula $2^{-\Delta\Delta Ct,}$ where;

$\Delta\Delta Ct = (CtDRA_{calibrator} - CtDRA_{test}) - (CtDQA_{calibrator} - CtDQA_{test})$.

Ct is defined as the point that the florescence rises above the baseline (Bodin, Beaune & Loriot 2005).

The efficiencies of the PCR amplification were calculated using a standard curve (Figure 5.14) giving values of 2.25 and 2.31, indicating similar efficiencies. Using the $2*2^{-\Delta\Delta Ct}$ method the copy number of each gene was calculated using animal 10814 as the control with only 2 copies of the DQA gene. The results of the qPCR copy number calculation are shown in Figure 5.15. The qPCR copy number agrees with the expected copy number for each of the animal samples (Table 5.1). From the qPCR animal 10814 was confirmed as having 2 copies of *DQA*, animals 10795 and 10775 were confirmed as having 3 copies and animals 2390, 2859, 2858 and10812 were confirmed as having 4 copies.

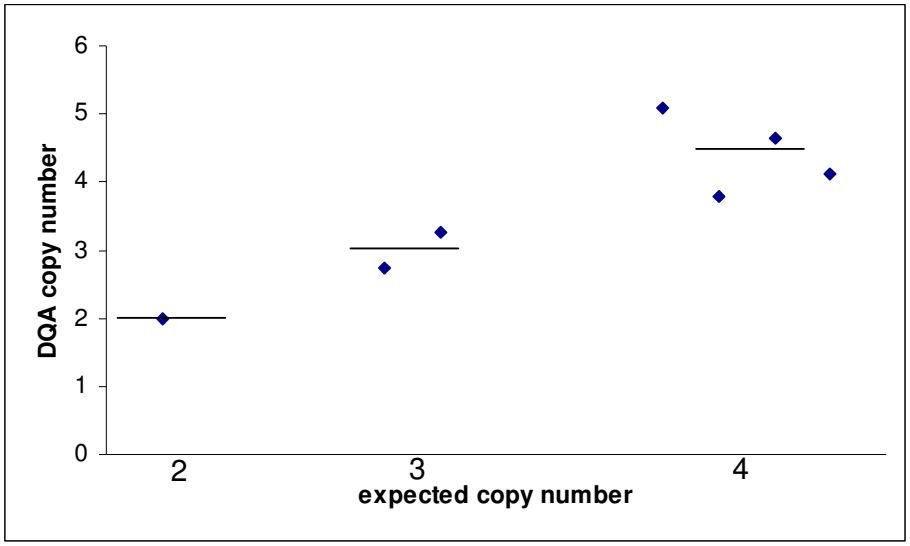


Figure 5.15 DQA copy number calculated using qPCR. The expected number of DQA copies generated from previous work (Table 5.1) and the calculated copy number using the $2*2^{-\Delta\Delta Ct}$ method.

5.7 Discussion

In this study two different approaches have been used to investigate the Class I region and the DQA genes. The DNA typing microarray used known information on the alleles of the DQA genes and the Class I genes to allow the deciphering of hybridisation patterns and allocation of alleles/haplotypes in the RoBoGen population. This technique however was only partially successful. The second approach was to utilise real-time qPCR as a quick and simple way to check for duplication of the DQA genes, which proved to be successful, although more thorough testing would be required.

The microarray technique was used to allocate alleles for the DQA genes and for Class I haplotypes. The success of a microarray is dependent upon the alleles which are present in the test population being represented on the array. These arrays were originally designed for the Holstein breed of cattle and for bison, which may account for some of the difficulties in the typing of the RoBoGen cross population. Nonetheless, it was still possible to identify the DQA and Class I alleles present in the majority of animals tested.

The Class I microarray used the known Class I haplotypes which were originally gained from older typing techniques (**Chapter 2**). However there is still much which is unknown about the organisation and structure of the Class I region in cattle, thus creating difficulty in assigning alleles/haplotypes within this region (Ellis et al. 1999). The array used both exon 2 and exon 3 sequences to identify 'haplotypes'. The majority of animals were assigned with at least 1 haplotype (Appendix D.8), but it is possible that there were more Class I genes present in the Charolais breed which are not

represented on the array. Also there could be difficulties when using the microarray on other breeds of cattle, as the defined Class I 'haplotypes' might not be the same.

It was intended that the information on spot intensity and probe identity would allow for the allocation of alleles. However, the attempted automation of microarray analysis was unsuccessful for several reasons. The microarray scanner used at the Ark Genomics facility is primarily for use on expression microarrays using dyes of Cy3 (green) and Cy5 (red). The wavelength was set to read at the Cy3 setting (532 nm). However, the optimum wavelength for the Alex Fluor 555 is 555 nm which unfortunately resulted in the quality of the scanned image being poor (Figure. 5.6). This in turn meant that the GenePix .gal file had difficulty identifying the positive spots so that manual selection of each spot was required in the majority of cases. Another problem with the automation was the variation in the original printing of the array, as each spot was not of uniform size, or in orientation to one another. This again resulted in a failure of the software to identified positive spots. The substantial differences in the area of the spots and irregularities in the spot quality meant that the average intensity over the entire spot would not be an accurate measure of intensity.

Once the spots were assigned an intensity value, it was intended that a program would be designed to automatically sort and allocate alleles. The Excel file was manipulated so that only spots/probes with high intensity 'positive' values were displayed. The probes which were listed positive were organised into the corresponding alleles. However, due to the problems in measuring the intensity values and the fact that the cut-off 'high' value was different between each slide, the computer algorithm failed to be applicable across all slides.

As the attempt to automate the analysis failed, all slide .jpeg images were emailed to Dr. Chris Davies to allow for subjective analysis and identification of alleles. The identification was done in conjugation with the known *DRB3* alleles and previous experience in reading the hybridisation patterns which assisted in *DQA* allele allocation.

The problems encountered in the typing of the animals using the DQA arrays were, for the most part, due to the technology. The hybridisation of the PCR products to the probes appears to have been successful; however, the analysis of the slides posed many problems. The scanning of the slides resulted in poor quality images which consequently led to an inability to visualise the positive probes. Manual assignment of positive probes was also difficult due to the poor quality of the scanned images. The final allele assignment method was determined in conjunction with the known *DRB3* alleles (**Chapter 2**), and experience in 'reading' the hybridisation patterns although it still is rather subjective. Whilst this did provide some information on the *DQA* alleles it was very time consuming and would be difficult to utilise in large population studies.

Despite the problems in the arrays it was possible to hypothesise the Class I-DRB3 haplotypes in the RoBoGen herd from the results. The typing of the MHC Class I region revealed that Class I haplotypes were linked with the *DRB3* alleles, for example *DRB3*2707* has Class I haplotype A12 in 34 animals (Table 5.3). The *A12:DRB3*2707* haplotype also is found with *DQA*0302* in 6 animals. The allele *DRB3*2707* is one of most frequent within the entire herd as 3 of the 4 founding sires had this allele, and therefore it is likely that the Charolais founding sires had the A12 Class I haplotype.

Real-time quantitative PCR has primarily been used in the study of gene expression but it has been adapted to investigate copy number variation (CNV) in a number of specific genes (Bodin, Beaune & Loriot 2005) and is a viable alternative to Southern blot and *in situ* hybridisation techniques (Hoebeeck, Speleman & Vandesompele 2007). In the present study, real-time qPCR was utilised to investigate the duplication in the *BoLA-DQA* genes. The duplication in these genes is a unique attribute of cattle (ruminants) (Ellis 2004), and is likely to improve the immune response by increasing the number of possible DQ molecules (Glass, Oliver & Russell 2000). The duplication of the DQ genes also allows for inter- and intra-haplotype paring of the alpha and beta chains, increasing the number of possible peptides which can be presented to T cells (Norimine & Brown 2005). Together with the high number of polymorphisms observed in these genes, it would indicate that the duplication of *DQA* and *DQB* would be advantageous. The duplication of the genes improves the level of resistance to mastitis (Park et al. 2004) and therefore might prove to be of benefit for a general level of disease resistance.

In this study I have developed a TaqMan real-time qPCR assay for the quantification of the BoLA-DQA genes. The DQA genes are highly polymorphic with 44 different alleles identified to date (http://www.ebi.ac.uk/ipd/mhc/bola/). For this reason it was problematic to design qPCR primers that lie in exon 2. Instead, the less polymorphic exon 3 was chosen. The main difficulty in using exon 3 for the designing of primer and probes for assay was the lack of sequence information available. Indeed, it was found that the sequence information was incorrect, with a C instead of a G in the reverse primer region. Nonetheless successful amplification of exon 3 was achieved after re-designing the primers to produce specific PCR products. *DRA* was chosen as a control

gene, as it is monomorphic and therefore easier to design suitable primers and probes as well as only being represented once on each chromosome. The qPCR assay allowed discrimination between the 2, 3 and 4 copies of DQA in each of the samples tested. Further validation on additional animals is necessary so that the qPCR method can be tested for accuracy and robustness.

The two techniques described in this chapter have been developed to increase the information on the Class I and DQA genes. The microarray typing has been utilised in typing and functional studies of the MHC genes (Park et al. 2004; Traul et al. 2005). The microarray technique could be further improved through greater levels of automation which might reduce the need for human analysis. The qPCR technique requires further testing for robustness, but could provide a simple and quick way of testing for duplication in the DQ genes. The qPCR assay could be used in functional studies with an aim to see if duplication increases disease resistance (further discussed in **Chapter 6**).

6 General discussion

Infectious disease has a substantial impact both on the economy of the livestock industry and the welfare of the animals. As it is now possible to further our understanding of the genetics underlying immune response to infections, we can start to utilise this information to design safer and more efficacious vaccines. It also opens up the possibility of breeding animals for increased disease resistance.

The alleles of *BoLA DRB3* have been previously reported to have significant associations with immune responses (**Chapter 1,** Table 1.1). *DRB3* is highly polymorphic and to date 106 alleles have been reported (http://www.ebi.ac.uk/ipd/mhc/bola/). This project was focussed on *DRB3* as it was highly probable that due to the function of the DR molecule, associations would be discovered in relation to the immune traits which were measured. In addition, it was feasible to accurately assign alleles to heterozygous animals.

The major resource for this PhD project was a large cattle cross population (RoBoGen herd) which was initially created for the study of production traits and locating possible QTLs. The production measurements and immune phenotypes were taken previous to the start of this project but included the response to immunisation with a peptide derived from FMDV, the IgG response to two respiratory disease vaccines, and the PBMC proliferation to *Staph. aureus*. Previous PhD projects had already found a strong sire-based genetic effect in the response to the respiratory vaccines (O'Neill et al. 2006; O'Neill 2006) and the PBMC proliferation response to *Staph. aureus* (Young 2002). This PhD study was unique however as its aim was to assess the associations between

polymorphisms present in BoLA genes and the previously measured immune phenotypes.

6.1 Overview

6.1.1 Key findings

The main findings from each of the 5 chapters are summarized below.

Chapter 2

- Improving a sequence-based typing method for BoLA- *DRB3*

Chapter 3

- Six *DRB3* alleles and seven PBC positions had significant associations with IgG1-specific response to FMDV15
- Three *DRB3* alleles and twelve PBC positions had significant associations with IgG2-specific response to FMDV15
- Three *DRB3* alleles and six PBC positions had significant associations with T cell proliferative response to FMDV15
- Two *DRB3* alleles and five PBC positions had significant associations with T cell proliferative response to ConA
- Three *DRB3* alleles and fifteen PBC positions had significant associations with IFN-γ production in response to FMDV15
- Three *DRB3* alleles and ten PBC positions had significant associations with IFN-γ production in response to ConA

Chapter 4

- Four *DRB3* alleles and six PBC positions had significant associations with IgG response to vaccination against BRSV
- Five *DRB3* alleles and five PBC positions had significant associations with IgG response to vaccination against BHV-1
- No *DRB3* alleles and four PBC positions had significant associations with IgG response to vaccination against PIV-3
- *DRB3* alleles and PBC positions had significant association with PMBC proliferation in response to *Staph. aureus* and PHA

Chapter 5

- Typing of MHC Class I haplotypes using a DNA microarray
- Typing of *DQA* alleles using a DNA microarray both at Washington State University and at the Roslin Institute
- Design of a novel qPCR technique for identifying duplication in *DQA* genes

In this PhD study a substantial number of significant associations between polymorphisms of the *DRB3* gene and the specific pocket positions within the PBC and the measured immune phenotypes were detected. The results when taken together are suggestive that the *DRB3* gene is important for the responses to vaccination. Moreover the specific amino acids within the PBC have significant associations with the humoral as well as cellular response with all the measured immune parameters.

In addition, as part of the overall project, typing of the DQA and Class I genes was undertaken (**Chapter 5**). There has been great difficulty with the typing of these genes

due to the high level of complexity. The *DQA* genes are duplicated in half the known haplotypes and are polymorphic (Andersson & Rask 1988; Ballingall, Luyai & Mckeever 1997). Using a sequence-based typing (SBT) method is difficult due to problems with allele identification. However, Takeshima et al. (2007) used SBT to type only at the *DQA1* locus. The number of Class I genes varies with haplotype, which has limited the research. Nonetheless, some alleles have been allocated to loci (Birch et al. 2006). These hurdles have restricted the study of these genes even though the polymorphisms in both sets of genes are likely to have a role in the mounting of an effective immune response. The animals of the RoBoGen herd had not been typed at either the *DQA* or Class I loci and so as part of the study investigating the BoLA region an attempt was made to distinguish their alleles using a typing microarray. In **Chapter 5** a novel qPCR technique was designed for the quantification of the number of *DQA* alleles, as the duplication of *DQ* gene may be of benefit to the immune response.

This project was part of a larger QTL investigation into production traits. The two breeds were chosen with the assumption that genes involved in either dairy or beef production would be fixed in the founder populations. However, it would be unlikely that those genes involved in the immune response would be fixed and instead has provided an opportunity to investigate the role of the *BoLA* genes in a heterogeneous population.

This project was constrained by a number of factors, including the original design of the test population. Unfortunately the RoBoGen herd had a poor design as there were only 4 founding Charolais sires crossed with 111 Holstein dams, with some dams having multiple offspring through embryo transfer. This resulted in *DRB3*2707* being there most frequent allele as it was derived from 3 of the 4 founding Charolais sires. Little is

known about the frequencies of *DRB3* alleles in breeds other than Holsteins, as the majority of the typing has been carried out on this breed (Sigurdardottir et al. 1992; Davies et al. 1997). Nonetheless even though the RoBoGen population had only 22 alleles some significant associations were found. It would have been interesting to have a higher number of founding sires or to have female Charolais crossed with male Holstein-Friesians which may have led to a more diverse range of *DRB3* alleles, although this might have reduced the overall significance of the data.

The FMDV15 peptide was used on the herd for a variety of reasons. It was a peptide to which the animals would have no prior exposure and therefore there would be no maternal antibodies. Thus it was considered that immunisation with the peptide would facilitate assessment of the role of BoLA genes on both the primary and secondary immune responses. The use of peptide/synthetic vaccines is a real commercial possibility and so a measure of the impact that the *DRB3* alleles have on the response to such a potential vaccine would be useful, however it has to be noted that to-date there are no commercial peptide vaccines in use for humans. Even though the peptide is only a 40-mer there are still high levels of complexity seen in both the response and in the *DRB3* associations observed. Those alleles which were significantly associated with the humoral and cellular responses are different, for example the **1601* allele which is significant for both IgG1 and IgG2 did not have any significance for T cell proliferation, indicating that the mechanisms directing the immune response are complex, and that a deeper understanding would be required to design efficacious vaccines. Although the FMDV15 peptide is a useful tool for exploring the BoLA associations with immune response it is not a viable commercial vaccine, and indeed it is unlikely that FMDV peptide vaccines would ever induce the protection needed to be used prophylactically.

The efficacy of the Imuresp vaccine (as addressed in **Chapter 4)** to induce an antibody response is not very convincing, with a low response for both anti-PIV3 IgG and anti-BHV1 IgG. Thus the relevance of the significant *DRB3* alleles to response to these pathogens is unclear, and may instead represent associations with clearance of maternal antibody or natural exposure. The contributing factor of circulating maternal antibodies also may have had an impact on the results from the Rispoval vaccine IgG measurements (O'Neill et al. 2006). It may have been possible to find more associations with the respiratory vaccines without the presence of the maternal antibodies.

There is currently no literature on the impact of the polymorphisms of the BoLA genes on commercially available vaccines such as those described in **Chapter 4**. As the interaction between the adaptive immune system and a vaccine is crucial for vaccine efficacy, an understanding of the role of the MHC molecules is vital. Firstly, this study highlights that the alleles of the *DRB3* gene are associated with the production of antibodies to a vaccine and secondly, it indicates that the PBC pockets would appear to have a substantial role.

It is very unlikely that the microarrays described in **Chapter 5** could ever realistically be implemented on a large population for typing at any of the BoLA loci. The identification of the *DQA* alleles was close to impossible if the *DRB3* alleles weren't known, in addition this is based on the assumption that the *DQA-DRB3* haplotypes were the same in all breed of cattle, which has not been proven. In the Class I microarrays it proved difficult to define haplotypes without extensive knowledge of the hybridisation patterns.

6.2 Further Research

Since the start of this project, there have been a number of advances in the available technologies. The SBT technique as described in **Chapter 2** could be further improved by using newer high through-put sequencing techniques, such as pyrosequencing which has been used for the genotyping of complex genes and regions (Babik et al. 2009). Indeed pyrosequencing methodology has already been implemented for the complex Class I region of macaques (Wiseman et al. 2009). The sequencing and allele allocation of the Class I and Class II genes could both be improved by the use of pyrosequencing. However the complexity of gene duplication in the DQ genes and the complexity of the Class I region may still pose difficulties in accurately assigning haplotypes.

An original aim of this project was to define haplotypes over the entire BoLA region using single nucleotide polymorphism (SNP) markers (described in **Chapter 1**). The use of SNP haplotypes has been implemented extensively in humans (Gibbs et al. 2003). It would be valuable to define haplotypes in the BoLA region as they could be used to investigate for associations between haplotypes and/or individual SNPs with immune phenotypes. However to establish a SNP haplotyping method, it was necessary to first establish the position of the genes within the BoLA region. A map was produced *in silico* using BLAST information with human and bovine MHC genes (**Chapter 1**) and the Btau 3 assembly, with the intention of identifying the BAC (bacterial artificial chromosomes) clones used in the assembly of the genome which contained BoLA genes. However, this region has high levels of duplication and gaps in the bovine assembly, which resulted in low confidence in the order of the clones. The BAC clones need to be in the correct order so that the positions of SNPs, and therefore the haplotypes could be correctly allocated.

The results presented in **Chapter 3** described the associations of the *BoLA DRB3* alleles and polymorphisms with the immune response to FMDV15 peptide. Many significant associations were noted between the *DRB3* alleles and IgG1, IgG2, IFN-γ and to a lesser extent T cell proliferation post-immunisation. In addition the pockets within the PBC also showed many highly significant associations.

A possible addition to the FMDV work would have been to test sera from high and low responders to the whole FMDV virus by ELISA and also by neutralisation tests. These would have to be carried out at a Category 4 facility such as Institute for Animal Health (Pirbright). This would allow any correlations between neutralising antibodies and the *DRB3* alleles to be tested. To further this research a challenge study with FMDV after the immunisation would have provided information on correlations between the high responders in **Chapter 3** and those which demonstrated protection. However challenge facilities within the UK are very limited in terms of the number of animals that can be accommodated and instead typing animals from an FMDV endemic country could potentially provide valuable data.

The cellular and humoral phenotypes were measured in response to the peptide and different alleles were associated with each. A future project would be to select those animals positive for alleles which were associated with a high/or low response to FMDV15 and challenge them with the live virus.

The binding of the peptide fragments to a variety of high and low responders' *DRB3* alleles could be carried out through purification of MHC molecules and a binding assay (Haghparast et al. 2000). This could be then carried through to the crystal modelling of both the peptide fragment and DR molecules, although this would be costly and require

a vast amount of work. Nonetheless understanding the binding of peptide to DR molecules may assist in the design of future vaccines.

A future project for the extension of the research in **Chapter 4** could be to investigate T cell proliferation in response to the vaccines and associations with *DRB3* alleles, as there were low responses measured for both anti-PIV3 and BHV1 IgG1. Again the use of binding assays as described above could be implemented and peptide vaccines could be used to produce a stronger response. A further way to develop the *Staph. aureus* assay would be to test whether those animals with alleles which were associated with a high/low PBMC proliferation response were more or less susceptible to mastitis.

The advancement of epitope prediction using bioinformatics is expanding the scope for vaccine research. Sequence information for cattle pathogens is readily available but the binding motifs are not yet available, therefore predicting T cell epitopes is not currently possible. In humans the use of computer algorithms to predict T cell epitopes has been used. This has been more successful in Class I molecules due to the relative ease as predicting the motifs in the bound peptides (Nielsen et al. 2008).

An important consideration in the use of peptide vaccines is that not all peptides will bind to MHC molecules for presentation, and even if peptide do bind it may not be functionally important e.g. the recognition of the spacer region of the FMDV peptide (Glass & Millar 1995). In addition, the binding of a peptide to an MHC molecule is only part of the required interactions to initiate an effective adaptive immune response. The bound peptide has to interact with the TCR on the T cells. This interaction would need to be sufficient to mount and effective adaptive immune response. Knowledge on T cell epitopes in cattle is limited, however it has been possible in the case of FMDV to

identify MHC restriction and anchor residues through lymphocyte proliferation assays and alanine substitutions in peptides (Gerner et al. 2009).

Several associations have been found between the *DRB3* alleles and PBC positions, and to the response to ConA. ConA is a mitogen and therefore should elicit a non-specific proliferation of T cells, and so these associations were not expected and are difficult to explain. A different T cell mitogen, such as PHA (**Chapter 5**) could have been used as there were no significant alleles or pockets identified in response to PHA.

The qPCR technique described in **Chapter 5** could potentially provide a quick and easy way to determine the copy number of the DQ genes. It would be useful to further investigate this technique on more animals to prove its robustness, and explore whether the duplications have an impact on the level of immune response as has been previously suggested (Glass, Oliver & Russell 2000; Park et al. 2004).

The advances in the qPCR technique may even pave the way to distinguish between DQ alleles using allele-specific TaqMan probes (Wang & Smith 2007). This would enable the testing of the theory of heterozygote advantage at these loci (Takeshima et al. 2008). The relative importance of the DR and the DQ molecules is unknown, with many of the associations being linked to the easier-to-type *DRB3* gene. Increased research into the DQ genes may begin to ascertain the importance of these genes for response to infection and vaccination.

It is not possible to fully quantify the role that the *DRB3* polymorphisms have in the response to vaccination without fully comprehending the haplotype structure of this region. The *DQ* genes are likely to be linked to *DRB3* and at least in the case of FMDV peptides (Glass, Oliver & Russell 2000) code for functional restriction elements.

Therefore haplotypes of *DRB3-DQA-DQB* may show stronger associations and appropriate SNPs in these genes distinguishing such haplotypes may provide markers for breeding for improved vaccine responders and/or disease resistance. Much is still unknown about BoLA haplotype structure across the whole region. This lack of information on the structure and organisation of the bovine MHC is hampering research and would ideally be resolved in the near future, possibly by sequencing the whole region using many animals.

Although the present study has clearly underlined the importance of the MHC region in determining immune responsiveness, the variation in response to vaccines and infectious pathogens is clearly polygenic (Hill 1998). Ongoing research into the response to BSRV vaccination and FMDV immunisation has shown many QTLs which lie over non-MHC regions as well as the MHC region on BTA23 (Leach et al. 2010).

Non-MHC genes will be having an impact on the responses discussed in this thesis. However, the data in **Chapters 3** and **4,** as well as that of Sharif et al (2000) and Yoshida et al (2009), indicate that the *DRB3* PBC is associated with immune response and taking it into consideration when designing vaccines would certainly be advisable. The amino acids within pocket 4 are especially significant, as in humans pocket 4 has been demonstrated to have a major influence on the binding of peptides in the DR molecule (Fu et al. 1995). In addition in cattle there have been a number of associations between immune response and pocket 4 (Sharif, Mallard, & Sargeant 2000; Baxter et al. 2009). Pocket 4 is centrally located and is entirely encoded by exon 2 of *DRB3.* It has also been demonstrated to be under positive selection (Takeshima et al. 2009).

For an effective vaccine an appropriate adaptive immune response and the generation of memory cells need to be provoked. Whilst the binding of vaccine peptides to MHC molecules is an important consideration, the TCR recognition of peptides as foreign is essential. The mechanisms by which the MHC:peptide complex interact with the TCR are still unknown, which would therefore limit the commercial possibilites of designing peptide vaccines.

6.3 Conclusion

In conclusion this thesis has confirmed that the BoLA genes have an important role in the bovine immune response, and that the *DRB3* polymorphisms have significant associations with both the humoral and cellular responses to a variety of stimuli. In addition this project has investigated the associations of *DRB3* alleles with response to commercially available viral vaccines, which have not previously been published. As a whole, the data presented here give an insight into the role of polymorphism in *DRB3* in the immune response, which could be utilised to improve vaccine design or breeding for disease resistance. Knowledge of these genes could be further expanded and utilised not only in livestock but in other species.

However, the BoLA region is very complex, with high levels of polymorphism and gene duplications. It would be beneficial to further research these genes and endeavour to fully unravel the complexities and polymorphisms to enable the design of better vaccines and allow for the breeding of cattle with higher resistance to infectious disease.

References

Aarestrup, F. M., Jensen, N. E., & Ostergard, H. 1995, "Analysis of associations between major histocompatibility complex (BoLA) Class-I haplotypes and subclinical mastitis of dairy-cows", *Journal of Dairy Science*, vol. 78, no. 8, pp. 1684-1692.

Allen, A. R., Minozzi, G., Glass, E. J., Skuce, R. A., McDowell, S. W. J., Woolliams, J. A., & Bishop, S. C. 2010, "Bovine tuberculosis: the genetic basis of host susceptibility", *Proceedings of the Royal Society B: Biological Sciences*, vol. 277, no. 1695, pp. 2737-2745.

Amadori, M., Archetti, I. L., Verardi, R., & Berneri, C. 1992, "Isolation of Mononuclear Cytotoxic-Cells from Cattle Vaccinated Against Foot-And-Mouth-Disease", *Archives of Virology*, vol. 122, no. 3-4, pp. 293-306.

Andersson, L. & Rask, L. 1988, "Characterization of the MHC Class-II region in cattle - the number of DQ genes varies between haplotypes", *Immunogenetics*, vol. 27, no. 2, pp. 110-120.

Anonymous 2003, *Off J Eur Union*, vol. Council Directive 2003/85/EC, no. 46:L306.

Antonis, A. F. G., Schrijver, R. S., Daus, F., Steverink, P. J. G. M., Stockhofe, N., Hensen, E. J., Langedijk, J. P. M., & van der Most, R. 2003, "Vaccine-induced immunopathology during bovine respiratory syncytial virus infection: Exploring the parameters of pathogenesis", *Journal of Virology*, vol. 77, no. 22, pp. 12067-12073.

Babbitt, B. P., Allen, P. M., Matsueda, G., Haber, E., & Unanue, E. R. 1985, "Binding of Immunogenic Peptides to Ia Histocompatibility Molecules", *Nature*, vol. 317, no. 6035, pp. 359-361.

Babik, W., Taberlet, P., Ejsmond, M. J., & Radwan, J. 2009, "New generation sequencers as a tool for genotyping of highly polymorphic multilocus MHC system", *Molecular Ecology Resources*, vol. 9, no. 3, pp. 713-719.

Babiuk, S., Horseman, B., Zhang, C., Bickis, M., Kusalik, A., Schook, L. B., Abrahamsen, M. S., & Pontarollo, R. 2007, "BoLA Class I allele diversity and polymorphism in a herd of cattle", *Immunogenetics*, vol. 59, no. 2, pp. 167-176.

Bacon, L. D., Witter, R. L., & Silva, R. F. 2001, "Characterization and experimental reproduction of peripheral neuropathy in White Leghorn chickens", *Avian Pathology*, vol. 30, no. 5, pp. 487-499.

Ballingall, K. T., Luyai, A., & Mckeever, D. J. 1997, "Analysis of genetic diversity at the DQA loci in African cattle: Evidence for a BoLA-DQA3 locus", *Immunogenetics*, vol. 46, no. 3, pp. 237-244.

Ballingall, K. T., Luyai, A., Rowlands, G. J., Sales, J., Musoke, A. J., Morzaria, S. P., & Mckeever, D. J. 2004, "Bovine leukocyte antigen major histocompatibility complex Class II DRB3*2703 and DRB3*1501 alleles are associated with variation in levels of protection against *Theileria parva* challenge following immunization with the sporozoite p67 antigen", *Infection and Immunity*, vol. 72, no. 5, pp. 2738-2741.

Banchereau, J., & Steinman, R.M., 1998 "Dendritic cells and the control of immunity" *Nature*, vol. 392, pp. 245-252

Band, M. R., Larsen, H. J., Womack, J. E., & Lewin, H. A. 1998, "A Radiation Hybrid Map of BTA23: Identification of a Chromosomal Rearrangement Leading to Separation of the Cattle MHC Class II Subregions", *Genomics*, vol. 53, no. 3, pp. 269-275.

Bates, T. W., Carpenter, T. E., & Thurmond, M. C. 2003, "Benefit-cost analysis of vaccination and preemptive slaughter as a means of eradicating foot-and-mouth disease", *American Journal of Veterinary Research*, vol. 64, no. 7, pp. 805-812.

Baumgartner, C. K., Ferrante, A., Nagaoka, M., Gorski, J., & Malherbe, L. P. 2010, "Peptide-MHC Class II Complex Stability Governs CD4 T Cell Clonal Selection", *Journal of Immunology*, vol. 184, no. 2, pp. 573-581.

Baxter, R., Craigmile, S. C., Haley, C., Douglas, A. J., Williams, J. L., & Glass, E. J. 2009, "BoLA-DR peptide binding pockets are fundamental for foot-and-mouth disease virus vaccine design in cattle", *Vaccine*, vol. 28, no. 1, pp. 28-37.

Baxter, R., Hastings, N., Law, A., & Glass, E. J. 2008, "A rapid and robust sequence-based genotyping method for BoLA-DRB3 alleles in large numbers of heterozygous cattle", *Animal Genetics*, vol. 39, no. 5, pp. 561-563.

Belov, K., Deakin, J. E., Papenfuss, A. T., Baker, M. L., Melman, S. D., Siddle, H. V., Gouin, N., Goode, D. L., Sargeant, T. J., Robinson, M. D., Wakefield, M. J., Mahony, S., Cross, J. G. R., Benos, P. V., Samollow, P. B., Speed, T. P., Graves, J. A. M., & Miller, R. D. 2006, "Reconstructing an ancestral mammalian immune supercomplex from a marsupial major histocompatibility complex", *Plos Biology*, vol. 4, no. 3, pp. 317-328.

Berinstein, A., Roivainen, M., Hovi, T., Mason, P. W., & Baxt, B. 1995, "Antibodies to the vitronectin receptor (integrin alpha(V)beta(3)) inhibit binding and infection of Foot-and-Mouth-disease virus to cultured-cells", *Journal of Virology*, vol. 69, no. 4, pp. 2664-2666.

Bernoco, D., Lewin, H. A., Andersson, L., Arriens, M. A., Byrns, G., Cwik, S., Davies, C. J., Hines, H. C., Leibold, W., Lie, O., Meggiolaro, D., Oliver, R. A., ostergard, H., Spooner, R. L., Stewarthaynes, J. A., Teale, A. J., Templeton, J. W., & Zanotti, M. 1991, "Joint Report of the 4th International Bovine Lymphocyte Antigen (BoLA)

Workshop, East Lansing, Michigan, USA, 25 August 1990", *Animal Genetics*, vol. 22, no. 6, pp. 477-496.

Berzofsky, J. A., Ahlers, J. D., & Belyakov, I. M. 2001, "Strategies for designing and optimizing new generation vaccines", *Nature Reviews Immunology*, vol. 1, no. 3, pp. 209-219.

Bevan, M. J. 1976, "Cross-priming for a secondary cytotoxic response to minor H-antigens with H-2 congenic cells which do not cross-react in cytotoxic assay", *Journal of Experimental Medicine*, vol. 143, no. 5, pp. 1283-1288.

Birch, J., Murphy, L., MacHugh, N. D., & Ellis, S. 2006, "Generation and maintenance of diversity in the cattle MHC Class I region", *Immunogenetics*, vol. 58, no. 8, pp. 670-679.

Blackwell, J. M., Jamieson, S. E., & Burgner, D. 2009, "HLA and Infectious Diseases", *Clinical Microbiology Reviews*, vol. 22, no. 2, p. 370-+.

Bodin, L., Beaune, P. H., & Loriot, A. M. 2005, "Determination of cytochrome P450 2D6 (CYP2D6) gene copy number by real-time quantitative PCR", *Journal of Biomedicine and Biotechnology*, vol. 3, pp. 248-253.

Bondinas, G. P., Moustakas, A. K., & Papadopoulos, G. K. 2007, "The spectrum of HLA-DQ and HLA-DR alleles, 2006: a listing correlating sequence and structure with function", *Immunogenetics*, vol. 59, no. 7, pp. 539-553.

Bowland, S. L. & Shewen, P. E. 2000, "Bovine respiratory disease: Commercial vaccines currently available in Canada", *Canadian Veterinary Journal-Revue Veterinaire Canadienne*, vol. 41, no. 1, pp. 33-48.

Brinkmeyer-Langford, C., Childers, C. P., Fritz, K., Gustafson-Seabury, A., Cothran, M., Raudsepp, T., Womack, J. E., & Skow, L. C. 2009, "A high resolution RH map of the bovine major histocompatibility complex", *BMC Genomics*, vol. 10, pp. 182-192.

Brown, J. H., Jardetzky, T. S., Gorga, J. C., Stern, L. J., Urban, R. G., Strominger, J. L., & Wiley, D. C. 1993, "3-Dimensional structure of the human Class-II histocompatibility antigen HLA-DR1", *Nature*, vol. 364, no. 6432, pp. 33-39.

Brown, W. C., Rice-Ficht, A. C., & Estes, D. M. 1998, "Bovine type 1 and type 2 responses", *Veterinary Immunology and Immunopathology*, vol. 63, no. 1-2, pp. 45-55.

Bryson, D. G., Adair, B. M., McNulty, M. S., McAliskey, M., Bradford, H. E. L., Allan, G. M., Evans, R. T., & Forster, F. 1999, "Studies on the efficacy of intranasal vaccination for the prevention of experimentally induced parainfluenza type 3 virus pneumonia in calves", *Veterinary Record*, vol. 145, no. 2, pp. 33-39.

Burman, A., Clark, S., Abrescia, N. G. A., Fry, E. E., Stuart, D. I., & Jackson, T. 2006, "Specificity of the VP1 GH loop of foot-and-mouth disease virus for alpha v integrins", *Journal of Virology*, vol. 80, no. 19, pp. 9798-9810.

Burroughs, A. L., Morrill, J. L., Bostwick, J. L., Ridley, R. K., & Fryer, H. C. 1982, "Immune-responses of dairy-cattle to para-influenza-3 virus in intranasal infectious bovine rhinotracheitis-iarainfluenza-3 virus-vaccines", *Canadian Journal of Comparative Medicine-Revue Canadienne de Medecine Comparee*, vol. 46, no. 3, pp. 264-266.

Burton, D. R. 2002, "Antibodies, viruses and vaccines", *Nat Rev Immunol*, vol. 2, no. 9, pp. 706-713.

Call, D. R., Chandler, D. P., & Brockman, F. 2001, "Fabrication of DNA microarrays using unmodified oligonucleotide probes", *Biotechniques*, vol. 30, no. 2, p. 368-+.

Capozzo, A. V. E., Periolo, O. H., Robiolo, B., Seki, C., LaTorre, J. L., & Grigera, P. R. 1997, "Total and isotype humoral responses in cattle vaccinated with foot and mouth disease virus (FMDV) immunogen produced either in bovine tongue tissue or in BHK-21 cell suspension cultures", *Vaccine*, vol. 15, no. 6-7, pp. 624-630.

Cardenas, C., Ortiz, M., Balbin, A., Villaveces, J. L., & Patarroyo, M. E. 2005, "Allele effects in MHC-peptide interactions: A theoretical analysis of HLA-DR beta 1*0101-HA and HLA-DR beta 1*0401-HA complexes", *Biochemical and Biophysical Research Communications*, vol. 330, no. 4, pp. 1162-1167.

Carrington, M., Nelson, G. W., Martin, M. P., Kissner, T., Vlahov, D., Goedert, J. J., Kaslow, R., Buchbinder, S., Hoots, K., & O'Brien, S. J. 1999, "HLA and HIV-1: Heterozygote advantage and B*35-Cw*04 disadvantage", *Science*, vol. 283, no. 5408, pp. 1748-1752.

Childers, C. P., Newkirk, H. L., Honeycutt, D. A., Ramlachan, N., Muzney, D. M., Sodergren, E., Gibbs, R. A., Weinstock, G. M., Womack, J. E., & Skow, L. C. 2006, "Comparative analysis of the bovine MHC Class IIb sequence identifies inversion breakpoints and three unexpected genes", *Animal Genetics*, vol. 37, no. 2, pp. 121-129.

Childerstone, A. J., Cedillo-Baron, L., Foster-Cuevas, M., & Parkhouse, R. M. E. 1999, "Demonstration of bovine CD8(+) T-cell responses to foot and mouth disease virus", *Journal of General Virology*, vol. 80, pp. 663-669.

Chowdhury, S. I. 1997, "Fine mapping of bovine herpesvirus 1 (BHV-1) glycoprotein C neutralizing epitopes by type-specific monoclonal antibodies and synthetic peptides", *Veterinary Microbiology*, vol. 58, no. 2-4, pp. 309-314.

Coelingh, K. J., Winter, C. C., Murphy, B. R., Rice, J. M., Kimball, P. C., Olmsted, R. A., & Collins, P. L. 1986, "Conserved epitopes on the hemagglutinin-neuraminidase proteins of human and bovine para-influenza type-3 viruses - nucleotide-sequence analysis of variants selected with monoclonal-antibodies", *Journal of Virology*, vol. 60, no. 1, pp. 90-96.

Collen, T. 1994, "Foot-and-mouth (Aphthovirus): Viral T cell epitopes," in *Cell-mediated immunity in ruminants*, B. M. L. Goddeeris & I. W. Morrison, eds., CRC press, pp. 173-190.

Collen, T., Dimarchi, R., & Doel, T. R. 1991, "A T cell epitope in VP1 of foot-and-mouth disease virus is immunodominant for vaccinated cattle", *The Journal of Immunology*, vol. 146, no. 2, pp. 749-755.

Cooke, G. S. & Hill, A. V. S. 2001, "Genetics of susceptibility to human infectious disease", *Nature Reviews Genetics*, vol. 2, no. 12, pp. 967-977.

Cornish-Bowden, A. 1985, "Nomenclature for incompletely specified bases in nucleic acid sequences: rcommendations 1984", *Nucleic Acids Research*, vol. 13, no. 9, pp. 3021-3030.

Court, R. A., Sitte, K., Opdebeeck, J. P., & East, I. J. 1998, "Mapping the T cell epitopes of the Babesia bovis antigen 12D3: implications for vaccine design", *Parasite Immunology*, vol. 20, no. 1, pp. 1-8.

Cresswell, P. 1994, "Assembly, transport, and function of MHC Class-II molecules", *Annual Review of Immunology*, vol. 12, pp. 259-293.

Crew, M. D. 1997, "Compilation of distinct HLA-A, -B and -C transmembrane and cytoplasmic domain-encoding sequences", *European Journal of Immunogenetics*, vol. 24, no. 6, pp. 443-449.

Cubillos, C., de la Torre, B. G., Jakab, A., Clementi, G., Borras, E., Barcena, J., Andreu, D., Sobrino, F., & Blanco, E. 2008, "Enhanced mucosal immunoglobulin a response and solid protection against foot-and-mouth disease virus challenge induced by a novel dendrimeric peptide", *Journal of Virology*, vol. 82, no. 14, pp. 7223-7230.

Cummings, C. A. & Relman, D. A. 2000, "Using DNA microarrays to study host-microbe interactions", *Emerging Infectious Diseases*, vol. 6, no. 5, pp. 513-525.

Davies, C. J., Andersson, L., Ellis, S. A., Hensen, E. J., Lewin, H. A., Mikko, S., MuggliCockett, N. E., vanderPoel, J. J., & Russell, G. C. 1997, "Nomenclature for factors of the BoLA system, 1996: Report of the ISAG BoLA Nomenclature Committee", *Animal Genetics*, vol. 28, no. 3, pp. 159-168.

Davies, C. J., Eldridge, J. A., Fisher, P. J., & Schlafer, D. H. 2006, "Evidence for expression of both classical and non-classical major histocompatibility complex Class I genes in bovine trophoblast cells", *American Journal of Reproductive Immunology*, vol. 55, no. 3, pp. 188-200.

Davies, C. J., Joosten, I., Andersson, L., Arriens, M. A., Bernoco, D., Bissumbhar, B., Byrns, G., Vaneijk, M. J. T., Kristensen, B., Lewin, H. A., Mikko, S., Morgan, A. L. G., MuggliCockett, N. E., Nilsson, P. R., Oliver, R. A., Park, C. A., vanderPoel, J. J., Polli, M., Spooner, R. L., & Stewart, J. A. 1994, "Polymorphism of bovine MHC Class-II genes - joint report of the fifth international bovine lymphocyte antigen (BoLA) workshop, Interlaken, Switzerland, 1-August-1992", *European Journal of Immunogenetics*, vol. 21, no. 4, pp. 259-289.

Davies, G. 2002, "The foot and mouth disease (FMD) epidemic in the United Kingdom 2001", *Comparative Immunology Microbiology and Infectious Diseases*, vol. 25, no. 5-6, pp. 331-343.

Davies, G., Genini, S., Bishop, S. C., & Giuffra, E. 2009, "An assessment of opportunities to dissect host genetic variation in resistance to infectious diseases in livestock", *Animal*, vol. 3, no. 3, pp. 415-436.

De Groot, A. S., Bosma, A., Chinai, N., Frost, J., Jesdale, B. M., Gonzalez, M. A., & Martin, W. 2001, "From genome to vaccine: in silico predictions, ex vivo verification", *Vaccine*, vol. 19, no. 31, pp. 4385-4395.

De Groot, A. S., Nene, V., Hegde, N. R., Srikumaran, S., Rayner, J., & Martin, W. 2003, "T cell epitope identification for bovine vaccines: an epitope mapping method for BoLA A-11", *International Journal for Parasitology*, vol. 33, no. 5-6, pp. 641-653.

Denis, M., Hanon, E., Rijsewijk, F. A. M., Kaashoek, M. J., van Oirschot, J. T., Thiry, E., & Pastoret, P. P. 1996, "The role of glycoproteins gC, gE, gI and gG in the induction of cell-mediated immune responses to bovine herpesvirus 1", *Veterinary Microbiology*, vol. 53, no. 1-2, pp. 121-132.

Di Palma, F., Archibald, S. D., Young, J. R., & Ellis, S. A. 2002, "A BAC contig of approximately 400 kb contains the classical class I major histocompatibility complex (MHC) genes of cattle", *European Journal of Immunogenetics*, vol. 29, no. 1, pp. 65-68.

Diaz, S., Echeverria, M. G., It, V., Posik, D. M., Rogberg-Munoz, A., Pena, N. L., Peral-Garcia, P., Vega-Pla, J. L., & Giovambattista, G. 2008, "Development of an ELA-DRA gene typing method based on pyrosequencing technology", *Tissue Antigens*, vol. 72, no. 5, pp. 464-468.

Dietz, A. B., Detilleux, J. C., Freeman, A. E., Kelley, D. H., Stabel, J. R., & Kehrli, M. E. 1997, "Genetic association of bovine lymphocyte antigen DRB3 alleles with immunological traits of Holstein cattle", *Journal of Dairy Science*, vol. 80, no. 2, pp. 400-405.

Dimarchi, R., Brooke, G., Gale, C., Cracknell, V., Doel, T., & Mowat, N. 1986, "Protection of cattle against foot-and-mouth disease by a synthetic peptide", *Science*, vol. 232, no. 4750, pp. 639-641.

Ellis, S. 2004, "The cattle major histocompatibility complex: is it unique?", *Veterinary Immunology and Immunopathology*, vol. 102, no. 1-2, pp. 1-8.

Ellis, S. A. & Ballingall, K. T. 1999, "Cattle MHC: evolution in action?", *Immunological Reviews*, vol. 167, pp. 159-168.

Ellis, S. A., Holmes, E. C., Staines, K. A., Smith, K. B., Stear, M. J., Mckeever, D. J., MacHugh, N. D., & Morrison, W. I. 1999, "Variation in the number of expressed MHC genes in different cattle Class I haplotypes", *Immunogenetics*, vol. 50, no. 5-6, pp. 319-328.

Ellis, S. A., Morrison, W. I., MacHugh, N. D., Birch, J., Burrells, A., & Stear, M. J. 2005, "Serological and molecular diversity in the cattle MHC Class I region", *Immunogenetics*, vol. 57, no. 8, pp. 601-606.

Endsley, J. J., Roth, J. A., Ridpath, J., & Neill, J. 2003, "Maternal antibody blocks humoral but not T cell responses to BVDV", *Biologicals*, vol. 31, no. 2, pp. 123-125.

Engelhard, V. H. 1994, "Structure of peptides associated with Class-I and Class-II MHC molecules", *Annual Review of Immunology*, vol. 12, pp. 181-207.

Erlich, H. A., Opelz, G., & Hansen, J. 2001, "HLA DNA typing and transplantation", *Immunity*, vol. 14, no. 4, pp. 347-356.

Estes, D. M. & Brown, W. C. 2002, "Type 1 and type 2 responses in regulation of Ig isotype expression in cattle", *Veterinary Immunology and Immunopathology*, vol. 90, no. 1-2, pp. 1-10.

Fitzpatrick, J. L., Logan, K. E., Young, F. J., Stear, M. J., Platt, D. J., & McGuirk, B. J. 1999, "Breeding cattle for mastitis resistance", Proceedings of the British Mastitis Conference (1999), Stoneleigh, UK, pp. 46-53.

Flajnik, M. F. & Kasahara, M. 2001, "Comparative genomics of the MHC: glimpses into the evolution of the adaptive immune system", *Immunity*, vol. 15, no. 3, pp. 351-362.

Fogg, M. H., Parsons, K. R., Thomas, L. H., & Taylor, G. 2001, "Identification of CD4(+) T cell epitopes on the fusion (F) and attachment (G) proteins of bovine respiratory syncytial virus (BRSV)", *Vaccine*, vol. 19, no. 23-24, pp. 3226-3240.

Frank, G. H. & Marshall, R. G. 1971, "Relationship of serum and nasal secretion-neutralizing antibodies in protection of calves against parainfluenza-3 virus", *American Journal of Veterinary Research*, vol. 32, no. 11, p. 1707-&.

Frank, S. A. 2002, "Experimental Evolution: Foot-and-Mouth Disease Virus," in *Immunology and Evolution of Infectious Disease.*, Princeton University Press.

Fraser, D. C., Craigmile, S., Campbell, J. D. M., Oliver, R. A., Brown, D. J., Russell, G. C., Spooner, R. L., & Glass, E. J. 1996, "Functional expression of a cattle MHC Class II DR-like antigen on mouse L cells", *Immunogenetics*, vol. 43, no. 5, pp. 296-303.

Fu, X. T., Bono, C. P., Woulfe, S. L., Swearingen, C., Summers, N. L., Sinigaglia, F., Sette, A., Schwartz, B. D., & Karr, R. W. 1995, "Pocket 4 of the HLA-DR (alpha, beta 1*0401) molecule is a major determinant of T cells recognition of peptide", *The Journal of Experimental Medicine*, vol. 181, no. 3, pp. 915-926.

Fulton, R. W., Briggs, R. E., Payton, M. E., Confer, A. W., Saliki, J. T., Ridpath, J. F., Burge, L. J., & Duff, G. C. 2004, "Maternally derived humoral immunity to bovine viral diarrhea virus (BVDV) 1a, BVDV1b, BVDV2, bovine herpesvirus-1, parainfluenza-3 virus bovine respiratory syncytial virus, Mannheimia haemolytica and Pasteurella

multocida in beef calves, antibody decline by half-life studies and effect on response to vaccination", *Vaccine*, vol. 22, no. 5-6, pp. 643-649.

Garboczi, D. N. & Biddison, W. E. 1999, "Shapes of MHC restriction", *Immunity*, vol. 10, no. 1, pp. 1-7.

Gaddum, R. M., Cook, R. S., Thomas, L. H., & Taylor, G. 1996, "Primary cytotoxic T-cell responses to bovine respiratory syncytial virus in calves", *Immunology*, vol. 88, no. 3, pp. 421-427.

Garcia-Briones, M. M., Russell, G. C., Oliver, R. A., Tami, C., Taboga, O., Carrillo, E., Palma, E. L., Sobrino, F., & Glass, E. J. 2000, "Association of bovine DRB3 alleles with immune response to FMDV peptides and protection against viral challenge", *Vaccine*, vol. 19, no. 9-10, pp. 1167-1171.

Gay, C. G., Zuerner, R., Bannantine, J. P., Lillehoj, H. S., Zhu, J. J., Green, R., & Pastoret, P. P. 2007, "Genomics and vaccine development", *Revue Scientifique et Technique-Office International des Epizooties*, vol. 26, no. 1, pp. 49-67.

Gelder, C. M., Lambkin, R., Hart, K. W., Fleming, D., Williams, O. M., Bunce, M., Welsh, K. I., Marshall, S. E., & Oxford, J. 2002, "Associations between human leukocyte antigens and nonresponsiveness to influenza vaccine", *The Journal of Infectious Diseases*, vol. 185, no. 1, pp. 114-117.

Gelhaus, A., Forster, B., Wippern, C., & Horstmann, R. D. 1999, "Evidence for an additional cattle DQA locus, BoLA-DQA5", *Immunogenetics*, vol. 49, no. 4, pp. 321-327.

Germain, R. N. 1994, "MHC-dependent antigen-processing and peptide presentation - providing ligands for T-lymphocyte activation", *Cell*, vol. 76, no. 2, pp. 287-299.

Gerner, W., Carr, B. V., Wiesmuller, K. H., Pfaff, E., Saalmuller, A., & Charleston, B. 2007, "Identification of a novel foot-and-mouth disease virus specific T-cell epitope with immunodominant characteristics in cattle with MHC serotype A31", *Veterinary Research*, vol. 38, no. 4, pp. 565-572.

Gerner, W., Hammer, S. E., Wiesmuller, K. H., & Saalmuller, A. 2009, "Identification of major histocompatibility complex restriction and anchor residues of foot-and-mouth disease virus-derived bovine T-cell epitopes", *Journal of Virology*, vol. 83, no. 9, pp. 4039-4050.

Gershwin, L. J. 2007, "Bovine respiratory syncytial virus infection: immunopathogenic mechanisms", *Animal Health Research Reviews*, vol. 8, no. 2, pp. 207-213.

Ghodke, Y., Joshi, K., Chopra, A., & Patwardhan, B. 2005, "HLA and disease", *European Journal of Epidemiology*, vol. 20, no. 6, pp. 475-488.

Gibbs, R. A., Belmont, J. W., Hardenbol, P., Willis, T. D., Yu, F. L., Yang, H. M., Ch'ang, L. Y., Huang, W., Liu, B., Shen, Y., Tam, P. K. H., Tsui, L. C., Waye, M. M. Y., Wong, J. T. F., Zeng, C. Q., Zhang, Q. R., Chee, M. S., Galver, L. M., Kruglyak, S., Murray, S. S., Oliphant, A. R., Montpetit, A., Hudson, T. J., Chagnon, F., Ferretti, V., Leboeuf, M., Phillips, M. S., Verner, A., Kwok, P. Y., Duan, S. H., Lind, D. L., Miller, R. D., Rice, J. P., Saccone, N. L., Taillon-Miller, P., Xiao, M., Nakamura, Y., Sekine, A., Sorimachi, K., Tanaka, T., Tanaka, Y., Tsunoda, T., Yoshino, E., Bentley, D. R., Deloukas, P., Hunt, S., Powell, D., Altshuler, D., Gabriel, S. B., Qiu, R. Z., Ken, A., Dunston, G. M., Kato, K., Niikawa, N., Knoppers, B. M., Foster, M. W., Clayton, E. W., Wang, V. O., Watkin, J., Gibbs, R. A., Belmont, J. W., Sodergren, E., Weinstock, G. M., Wilson, R. K., Fulton, L. L., Rogers, J., Birren, B. W., Han, H., Wang, H. G., Godbout, M., Wallenburg, J. C., L'Archeveque, P., Bellemare, G., Todani, K., Fujita, T., Tanaka, S., Holden, A. L., Lai, E. H., Collins, F. S., Brooks, L. D., Mcewen, J. E., Guyer, M. S., Jordan, E., Peterson, J. L., Spiegel, J., Sung, L. M., Zacharia, L. F., Kennedy, K., Dunn, M. G., Seabrook, R., Shillito, M., Skene, B., Stewart, J. G., Valle, D. L., Clayton, E. W., Jorde, L. B., Belmont, J. W., Chakravarti, A., Cho, M. K., Duster, T., Foster, M. W., Jasperse, M., Knoppers, B. M., Kwok, P. Y., Licinio, J., Long, J. C., Marshall, P. A., Ossorio, P. N., Wang, V. O., Rotimi, C. N., Royal, C. D. M., Spallone, P., Terry, S. F., Lander, E. S., Lai, E. H., Nickerson, D. A., Abecasis, G. R., Altshuler, D., Bentley, D. R., Boehnke, M., Cardon, L. R., Daly, M. J., Deloukas, P., Douglas, J. A., Gabriel, S. B., Hudson, R. R., Hudson, T. J., Kruglyak, L., Kwok, P. Y., Nakamura, Y., Nussbaum, R. L., Royal, C. D. M., Schaffner, S. F., Sherry, S. T., Stein, L. D., & Tanaka, T. 2003, "The International HapMap Project", *Nature*, vol. 426, no. 6968, pp. 789-796.

Glass, E. J., Baxter, R., Leach, R., & Taylor, G. 2010, "Bovine viral diseases - The role of host genetics," in *Breeding for Disease Resistance in Farm Animals*, 3rd edn, S. C. Bishop et al., eds., CAB International, pp. 88-140.

Glass, E. J. 2004, "Genetic variation and response to vaccines", *Animal Health Research Reviews*, vol. 5, no. 2, pp. 197-208.

Glass, E. J. & Millar, P. 1994, "Induction of effective cross-reactive immunity by FMDV peptides is critically dependent upon specific MHC-peptide-T cell-interactions", *Immunology*, vol. 82, no. 1, pp. 1-8.

Glass, E. J. & Millar, P. 1995, "Bovine T-cells preferentially recognize nonviral spacer epitopes in a putative FMDV vaccinal peptide", *Vaccine*, vol. 13, no. 2, pp. 225-229.

Glass, E. J., Oliver, R. A., Collen, T., Doel, T. R., Dimarchi, R., & Spooner, R. L. 1991, "MHC Class-II restricted recognition of FMDV peptides by bovine T-cells", *Immunology*, vol. 74, no. 4, pp. 594-599.

Glass, E. J., Oliver, R. A., & Russell, G. C. 2000, "Duplicated DQ haplotypes increase the complexity of restriction element usage in cattle", *Journal of Immunology*, vol. 165, no. 1, pp. 134-138.

Glass, E. J., Oliver, R. A., & Spooner, R. L. 1991, "Bovine T-Cells recognize antigen in association with MHC Class-II haplotypes defined by one-dimensional isoelectric-focusing", *Immunology*, vol. 72, no. 3, pp. 380-385.

Glass, E. J., Oliver, R. A., Williams, J. L. W., & Millar, P. 1992, "Alloreactive T-Cell recognition of bovine major histocompatibility complex Class-II products defined by one-dimensional isoelectric-focusing", *Animal Genetics*, vol. 23, no. 2, pp. 97-111.

Glezen, W. P. 2003, "Effect of maternal antibodies on the infant immune response", *Vaccine*, vol. 21, no. 24, pp. 3389-3392.

Gogev, S., Vanderheijden, N., Lemaire, M., Schynts, F., D'Offay, J., Deprez, I., Adam, M., Eloit, M., & Thiry, E. 2002, "Induction of protective immunity to bovine herpesvirus type 1 in cattle by intranasal administration of replication-defective human adenovirus type 5 expressing glycoprotein gC or gD", *Vaccine*, vol. 20, no. 9-10, pp. 1451-1465.

Gorer, P. A. 1937, "The genetic and antigenic basis of tumour transplantation", *The Journal of Pathology and Bacteriology*, vol. 44, no. 3, pp. 691-697.

Goris, N., Willems, T., Diev, V. I., Merkelbach-Peters, P., Vanbinst, T., Van der Stede, Y., Kraft, H. P., Zakharov, V. M., Borisov, V. V., Nauwynck, H. J., Haas, B., & De Clercq, K. 2008, "Indirect foot-and-mouth disease vaccine potency testing based on a serological alternative", *Vaccine*, vol. 26, no. 31, pp. 3870-3879.

Gorodezky, C., Alaez, C., Murguia, A., Rodriguez, A., Balladares, S., Vazquez, M., Flores, H., & Robles, C. 2006, "HLA and autoimmune diseases: Type 1 diabetes (T1D) as an example", *Autoimmunity Reviews*, vol. 5, no. 3, pp. 187-194.

Greenwood, D. L. V., Dynon, K., Kalkanidis, M., Xiang, S., Plebanski, M., & Scheerlinck, J. P. Y. 2008, "Vaccination against foot-and-mouth disease virus using peptides conjugated to nano-beads", *Vaccine*, vol. 26, no. 22, pp. 2706-2713.

Grubman, M. J. & Baxt, B. 2004, "Foot-and-mouth disease", *Clinical Microbiology Reviews*, vol. 17, no. 2, pp. 465-493.

Guan, P., Doytchinova, I. A., Zygouri, C., & Flower, D. R. 2003, "MHCPred: a server for quantitative prediction of peptideΓÇôMHC binding", *Nucleic Acids Research*, vol. 31, no. 13, pp. 3621-3624.

Guzman, E., Taylor, G., Charleston, B., Skinner, M. A., & Ellis, S. A. 2008, "An MHC-restricted CD8+ T-cell response is induced in cattle by foot-and-mouth disease virus (FMDV) infection and also following vaccination with inactivated FMDV", *Journal of General Virology*, vol. 89, no. 3, pp. 667-675.

Haghparast, A., Wauben, M. H. M., Grosfeld-Stulemeyer, M. C., van Kooten, P., & Hensen, E. J. 2000, "Selection of T-cell epitopes from foot-and-mouth disease virus reflects the binding affinity to different cattle MHC Class II molecules", *Immunogenetics*, vol. 51, no. 8-9, pp. 733-742.

Hall, T. A. 1999, "BioEdit: a user friendly biological sequence editor and analysis program for window 95/98/NT", *nucleic acids symposium series*, vol. 41, pp. 95-98.

Hameed, K. G. A., Sender, G., & Korwin-Kossakowska, A. 2008, "An association of BoLA alleles DRB3.2*16 and DRB3.2*23 with occurrence of mastitis caused by different bacterial species in two herds of dairy cows", *Animal Science Papers and Reports*, vol. 26, no. 1, pp. 37-48.

Hanotte, O., Ronin, Y., Agaba, M., Nilsson, P., Gelhaus, A., Horstmann, R., Sugimoto, Y., Kemp, S., Gibson, J., Korol, A., Soller, M., & Teale, A. 2003, "Mapping of quantitative trait loci controlling trypanotolerance in a cross of tolerant West African N'Dama and susceptible East African Boran cattle", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 13, pp. 7443-7448.

Hauser, P. M. 2004, "The development of a typing method for an uncultivable microorganism: the example of Pneumocystis jirovecii", *Infection, Genetics and Evolution*, vol. 4, no. 3, pp. 199-203.

Hayashi, F., Smith, K.D., Ozinsky, A., Hawn, T.R., Yi, E.C., Goodlett, D.R., Eng, J.K., Akira, S., Underhill, D.M., & Aderem, A. 2001 "The inate immune resposnse to bacterial flagellin is mediated by Toll-like receptor 5", *Nature*, vol. 410 pp.1099-1103.

Heemels, M. T. & Ploegh, H. 1995, "Generation, translocation, and presentation of MHC Class I-restricted peptides", *Annual Review of Biochemistry*, vol. 64, pp. 463-491.

Hegde, N. R., Deshpande, M. S., Godson, D. L., Babiuk, L. A., & Srikumaran, S. 1999, "Bovine lymphocyte antigen-A11-specific peptide motif as a means to identify cytotoxic T-lymphocyte epitopes of bovine herpesvirus 1", *Viral Immunology*, vol. 12, no. 2, pp. 149-161.

Hegde, N. R. & Srikumaran, S. 1996, "Prediction of potential cytotoxic T lymphocyte epitopes of bovine herpesvirus 1 based on allele-specific peptide motifs and proteolytic cleavage specificities", *Virus Genes*, vol. 13, no. 2, pp. 121-133.

Henderson, G., Perng, G. C., Nesburn, A. B., Wechsler, S. L., & Jones, C. 2004, "The latency-related gene encoded by bovine herpesvirus 1 can suppress caspase 3 and caspase 9 cleavage during productive infection", *Journal of Neurovirology*, vol. 10, no. 1, pp. 64-70.

Heringstad, B., Chang, Y. M., Gianola, D., & Osteras, O. 2008, "Short communication: Genetic analysis of respiratory disease in Norwegian Red calves", *Journal of Dairy Science*, vol. 91, no. 1, pp. 367-370.

Hill, A. V. S. 1998, "The immunogenetics of human infectious diseases", *Annual Review of Immunology*, vol. 16, pp. 593-617.

Hinkley, S., Ambagala, A. P. N., Jones, C. J., & Srikumaran, S. 2000, "A vhs-like activity of bovine herpesvirus-1", *Archives of Virology*, vol. 145, no. 10, pp. 2027-2046.

Hoebeeck, J., Speleman, F., & Vandesompele, J. 2007, "Real-time quantitative PCR as an alternative to Southern blot or fluorescence in situ hybridization for detection of gene copy number changes", *Methods in Molecular Biology*, vol. 353, pp. 205-226.

Holmes, E. C., Roberts, A. F. C., Staines, K. A., & Ellis, S. A. 2003, "Evolution of major histocompatibility complex Class I genes in cetartiodactyls", *Immunogenetics*, vol. 55, no. 4, pp. 193-202.

Horwood, P. F., Gravel, J. L., & Mahony, T. J. 2008, "Identification of two distinct bovine parainfluenza virus type 3 genotypes", *Journal of General Virology*, vol. 89, pp. 1643-1648.

Hussain, A. & Mohanty, S. B. 1984, "Cell-mediated-immunity of calves to bovine para-influenza-3 virus - leukocyte migration-inhibition and leukocyte aggregation response", *Zentralblatt fur Veterinarmedizin Reihe B-Journal of Veterinary Medicine Series B-Infectious Diseases Immunology Food Hygiene Veterinary Public Health*, vol. 31, no. 3, pp. 203-209.

Hussell, T., Georgiou, A., Sparer, T. E., Matthews, S., Pala, P., & Openshaw, P. J. M. 1998, "Host genetic determinants of vaccine-induced eosinophilia during respiratory syncytial virus infection", *Journal of Immunology*, vol. 161, no. 11, pp. 6215-6222.

Janssen, R., Bont, L., Siezen, C. L. E., Hodemaekers, H. M., Ermers, M. J., Doornbos, G., van't Slot, R., Wijmenga, C., Goeman, J. J., Kimpen, J. L. L., van Houwelingen, H. C., Kimman, T. G., & Hoebee, B. 2007, "Genetic susceptibility to respiratory syncytial virus bronchiolitis is predominantly associated with innate immune genes", *Journal of Infectious Diseases*, vol. 196, no. 6, pp. 826-834.

Jares, P. 2006, "DNA microarray applications in functional genomics", *Ultrastructural Pathology*, vol. 30, no. 3, pp. 209-219.

Jeffery, K. J. M. & Bangham, C. R. M. 2000, "Do infectious diseases drive MHC diversity?", *Microbes and Infection*, vol. 2, no. 11, pp. 1335-1341.

Jensen, P. E. 1998, "Antigen processing: HLA-DO - a hitchhiking inhibitor of HLA-DM", *Current Biology*, vol. 8, no. 4, p. R128-R131.

Joosten, I., Hensen, E. J., Sanders, M. F., & Andersson, L. 1990, "Bovine MHC Class-II restriction fragment length polymorphism linked to expressed polymorphism", *Immunogenetics*, vol. 31, no. 2, pp. 123-126.

Joosten, I., Teale, A. J., Vanderpoel, A., & Hensen, E. J. 1992, "Biochemical-evidence of the expression of 2 major histocompatibility complex Class-I genes on bovine peripheral-blood mononuclear-cells", *Animal Genetics*, vol. 23, no. 2, pp. 113-123.

Juleff, N., Windsor, M., Lefevre, E. A., Gubbins, S., Hamblin, P., Reid, E., McLaughlin, K., Beverley, P. C. L., Morrison, I. W., & Charleston, B. 2009, "Foot-and-mouth disease virus can induce a specific and rapid CD4(+) T-cell-independent

neutralizing and isotype Class-switched antibody response in naive cattle", *Journal of Virology*, vol. 83, no. 8, pp. 3626-3636.

Juliarena, M. A., Poli, M., Ceriani, C., Sala, L., Rodriguez, E., Gutierrez, S., Dolcini, G., Odeon, A., & Esteban, E. N. 2009, "Antibody response against three widespread bovine viruses is not impaired in Holstein cattle carrying bovine leukocyte antigen DRB3.2 alleles associated with bovine leukemia virus resistance", *Journal of Dairy Science*, vol. 92, no. 1, pp. 375-381.

Juliarena, M. A., Poli, M., Sala, L., Ceriani, C., Gutierrez, S., Dolcini, G., Rodriguez, E. M., Marino, B., Rodriguez-Dubra, C., & Esteban, E. N. 2000, "Association of BLV infection profiles with alleles of the BoLA-DRB3.2 gene", *Animal Genetics*, vol. 39, no. 4, pp. 432-438.

Kapil, S. & Basaraba, R. J. 1997, "Infectious bovine rhinotracheitis, parainfluenza-3, and respiratory coronavirus", *Veterinary Clinics of North America-Food Animal Practice*, vol. 13, no. 3, pp. 455-469.

Kappler, J. W., Roehm, N., & Marrack, P. 1987, "T-Cell Tolerance by Clonal Elimination in the Thymus", *Cell*, vol. 49, no. 2, pp. 273-280.

Kaufman, J., Milne, S., Gobel, T. W. F., Walker, B. A., Jacob, J. P., Auffray, C., Zoorob, R., & Beck, S. 1999, "The chicken B locus is a minimal essential major histocompatibility complex", *Nature*, vol. 401, no. 6756, pp. 923-925.

Kaur, G. & Mehra, N. 2009, "Genetic determinants of HIV-1 infection and progression to AIDS: immune response genes", *Tissue Antigens*, vol. 74, no. 5, pp. 373-385.

Kelley, J., Walter, L., & Trowsdale, J. 2005, "Comparative genomics of major histocompatibility complexes", *Immunogenetics*, vol. 56, no. 10, pp. 683-695.

Kelm, S. C., Detilleux, J. C., Freeman, A. E., Kehrli, M. E., Dietz, A. B., Fox, L. K., Butler, J. E., Kasckovics, I., & Kelley, D. H. 1997, "Genetic association between parameters of innate immunity and measures of mastitis in periparturient Holstein cattle", *Journal of Dairy Science*, vol. 80, no. 8, pp. 1767-1775.

Kimman, T. G., Westenbrink, F., & Straver, P. J. 1989, "Priming for local and systemic antibody memory responses to bovine respiratory syncytial virus: Effect of amount of virus, virus replication, route of administration and maternal antibodies", *Veterinary Immunology and Immunopathology*, vol. 22, no. 2, pp. 145-160.

Kitching, R. P. 2002, "Clinical variation in foot and mouth disease: cattle", *Revue Scientifique et Technique de l Office International des Epizooties*, vol. 21, no. 3, pp. 499-504.

Klungland, H., Sabry, A., Heringstad, B., Olsen, H. G., Gomez-Raya, L., Vage, D. I., Olsaker, I., Odegard, J., Klemetsdal, G., Schulman, N., Vilkki, J., Ruane, J., Aasland, M., Ronningen, K., & Lien, S. 2001, "Quantitative trait loci affecting clinical mastitis

and somatic cell count in dairy cattle", *Mammalian Genome*, vol. 12, no. 11, pp. 837-842.

Koppers-Lalic, D., Verweij, M. C., Lipinska, A. D., Wang, Y., Quinten, E., Reits, E. A., Koch, J., Loch, S., Rezende, M. M., Daus, F., Bienkowska-Szewczyk, K., Osterrieder, N., Mettenleiter, T. C., Heemskerk, M. H. M., Tampe, R., Neefjes, J. J., Chowdhury, S. I., Ressing, M. E., Rijsewijk, F. A. M., & Wiertz, E. J. H. J. 2008, "Varicellovirus UL49.5 proteins differentially affect the function of the transporter associated with antigen processing, TAP", *Plos Pathogens*, vol. 4, no. 5.

Kostia, S., Kantanen, J., Kolkkala, M., & Varvio, S. L. 1998, "Applicability of SSCP analysis for MHC genotyping: fingerprinting of Ovar-DRB1 exon 2 alleles from Finnish and Russian breeds", *Animal Genetics*, vol. 29, no. 6, pp. 453-455.

Kulberg, S., Heringstad, B., Guttersrud, O. A., & Olsaker, I. 2007, "Study on the association of BoLA-DRB3.2 alleles with clinical mastitis in Norwegian Red cows", *Journal of Animal Breeding and Genetics*, vol. 124, no. 4, pp. 201-207.

Laegreid, W. W., Heaton, M. P., Keen, J. E., Grosse, W. M., Chitko-McKown, C. G., Smith, T. P. L., Keele, J. W., Bennett, G., & Besser, T. E. 2002, "Association of bovine neonatal Fc receptor alpha-chain gene (FCGRT) haplotypes with serum IgG concentration in newborn calves", *Mammalian Genome*, vol. 13, pp. 704-710.

Laskowski, R. A., Macarthur, M. W., Moss, D. S., & Thornton, J. M. 1993, "Procheck - A Program to Check the Stereochemical Quality of Protein Structures", *Journal of Applied Crystallography*, vol. 26, pp. 283-291.

Leach, R. J., Craigmile, S. C., Knott, S. A., Williams, J. L., & Glass, E. J. Quantitative trait loci for variation in immune response to a Foot-and-Mouth Disease virus peptide. BMC Genetics. 2010.

Ledwidge, S. A., Mallard, B. A., Gibson, J. P., Jansen, G. B., & Jiang, Z. H. 2001, "Multi-primer target PCR for rapid identification of bovine DRB3 alleles", *Animal Genetics*, vol. 32, no. 4, pp. 219-221.

Lemaire, M., Meyer, G., Baranowski, E., Schynts, F., Wellemans, G., Kerkhofs, P., & Thiry, E. 2000, "Production of bovine herpesvirus type 1-seronegative latent carriers by administration of a live-attenuated vaccine in passively immunized calves", *Journal of Clinical Microbiology*, vol. 38, no. 44, p. 4233.

Lewin, H. A., Russell, G. C., & Glass, E. J. 1999, "Comparative organization and function of the major histocompatibility complex of domesticated cattle", *Immunological Reviews*, vol. 167, pp. 145-158.

Livak, K. J. & Schmittgen, T. D. 2001, "Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-[Delta][Delta]CT Method", *Methods*, vol. 25, no. 4, pp. 402-408.

Ljunggren, H. G. & Karre, K. 1990, "In search of the missing self - MHC molecules and NK cell recognition", *Immunology Today*, vol. 11, no. 7, pp. 237-244.

Longeri, M., Polli, M., Ponti, W., & Zanotti, M. 1993, "BoLA Class-I polymorphism and in-vitro immune-response to M-bovis-antigens", *Journal of Animal Breeding and Genetics-Zeitschrift fur Tierzuchtung und Zuchtungsbiologie*, vol. 110, no. 5, pp. 335-345.

Maillard, J. C., Berthier, D., Chantal, I., Thevenon, S., Sidibe, I., Stachurski, F., Belemsaga, D., Razafindraibe, H., & Elsen, J. M. 2003, "Selection assisted by a BoLA-DR/DQ haplotype against susceptibility to bovine dermatophilosis", *Genetics Selection Evolution*, vol. 35, p. S193-S200.

Maillard, J. C., Martinez, D., & Bensaid, A. 1996, "An amino acid sequence coded by the exon 2 of the BoLA DRB3 gene associated with a BoLA Class I specificity constitutes a likely genetic marker of resistance to dermatophilosis in Brahman zebu cattle of Martinique (FWI)", *Vector-Borne Pathogens: International Trade and Tropical Animal Diseases*, vol. 791, pp. 185-197.

Mckay, S. D., Schnabel, R. D., Murdoch, B. M., Matukumalli, L. K., Aerts, J., Coppieters, W., Crews, D., Dias, E., Gill, C. A., Gao, C., Mannen, H., Wang, Z. Q., Van Tassell, C. P., Williams, J. L., Taylor, J. F., & Moore, S. S. 2008, "An assessment of population structure in eight breeds of cattle using a whole genome SNP panel", *Bmc Genetics*, vol. 9, no. 37, pp. 1471-2156.

Meyer, G., Deplanche, M., & Schelcher, F. 2008, "Human and bovine respiratory syncytial virus vaccine research and development", *Comparative Immunology Microbiology and Infectious Diseases*, vol. 31, no. 2-3, pp. 191-225.

Nielsen, M., Lundegaard, C., Blicher, T., Peters, B., Sette, A., Justesen, S., Buus, S., & Lund, O. 2008, "Quantitative Predictions of Peptide Binding to Any HLA-DR Molecule of Known Sequence: NetMHCIIpan", *Plos Computational Biology*, vol. 4, no. 7.

Miltiadou, D. 2001, *Bovine MHC genotyping in correlation with immune response traits,* University of Edinburgh.

Miltiadou, D., Law, A. S., & Russell, G. C. 2003, "Establishment of a sequence-based typing system for BoLA-DRB3 exon 2", *Tissue Antigens*, vol. 62, no. 1, pp. 55-65.

Momburg, F. & Tan, P. 2002, "Tapasin--the keystone of the loading complex optimizing peptide binding by MHC Class I molecules in the endoplasmic reticulum", *Molecular Immunology*, vol. 39, no. 3-4, pp. 217-233.

Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A., & Coffman, R. L. 1986, "2 Types of Murine Helper T-Cell Clone .1. Definition According to Profiles of Lymphokine Activities and Secreted Proteins", *Journal of Immunology*, vol. 136, no. 7, pp. 2348-2357.

Mulcahy, G., Gale, C., Robertson, P., Iyisan, S., DiMarchi, R. D., & Doel, T. R. 1990, "Isotype responses of infected, virus-vaccinated and peptide-vaccinated cattle to foot-and-mouth disease virus", *Vaccine*, vol. 8, no. 3, pp. 249-256.

NADIS

2010

Internet Communication

www.hccmpw.org.uk/meadialibrary/pdf/279.pdf

Nasir, L., Ndiaye, M., Seely, C., & Stear, M. J. 1997, "Sequence polymorphism in the bovine major histocompatibility complex DQB loci", *Animal Genetics*, vol. 28, no. 6, pp. 441-445.

Newman, M. J., Truax, R. E., French, D. D., Dietrich, M. A., Franke, D., & Stear, M. J. 1996, "Evidence for genetic control of vaccine-induced antibody responses in cattle", *Veterinary Immunology and Immunopathology*, vol. 50, no. 1-2, pp. 43-54.

Norimine, J. & Brown, W. C. 2005, "Intrahaplotype and interhaplotype pairing of bovine leukocyte antigen DQA and DQB molecules generate functional DQ molecules important for priming CD4(+) T-lymphocyte responses", *Immunogenetics*, vol. 57, no. 10, pp. 750-762.

Norman, L. M., Hohenboken, W. D., & Kelley, K. W. 1981, "Genetic-differences in concentration of immunoglobulins-g1 and M in serum and colostrum of cows and in serum of neonatal calves", *Journal of Animal Science*, vol. 53, no. 6, pp. 1465-1472.

O'Neill, R. G. 2006, *Linking phenotype to genotype: a study of antibody responses to bovine respiratory virus vaccines in a crossbred cattle population.,* PhD, University of Glasgow.

O'Neill, R. G., Woolliams, J. A., Glass, E. J., Williams, J. L., & Fitzpatrick, J. L. 2006, "Quantative evaluation of genetic and environmental parameters determining antibody response induced by vaccination against bovine respiratory syncytial virus.", *Vaccine*, vol. 24, pp. 4007-4016.

Ohta, Y., Okamura, K., McKinney, E. C., Bartl, S., Hashimoto, K., & Flajnik, M. F. 2000, "Primitive synteny of vertebrate major histocompatibility complex Class I and Class II genes", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 9, pp. 4712-4717.

O'Shea, J. J. & Paul, W. E. 2010, "Mechanisms Underlying Lineage Commitment and Plasticity of Helper CD4(+) T Cells", *Science*, vol. 327, no. 5969, pp. 1098-1102.

Paape, M. J. & Guidry, A. J. 1977, "Inhibitory effects of milk components on polymorphonuclear leukocyte (PMN) phagocytosis and intracellular killing of *Staphylococcus-aureus*", *Journal of Dairy Science*, vol. 60, pp. 134-135.

Paape, M. J., Gwazdauskas, F. C., Guidry, A. J., & Weinland, B. T. 1981, "Concentrations of corticosteroids, leukocytes and immunoglobulins in blood and milk after administration of ACTH to lactating dairy-cattle -effects on phagocytosis of *Staphylococcus-aureus* by polymorphonuclear leukocytes", *American Journal of Veterinary Research*, vol. 42, no. 12, pp. 2081-2087.

Parra-Lopez, C., Calvo-Calle, J. M., Cameron, T. O., Vargas, L. E., Salazar, L. M., Patarroyo, M. E., Nardin, E., & Stern, L. J. 2006, "Major histocompatibility complex and T cell interactions of a universal T cell epitope from Plasmodium falciparum circumsporozoite protein", *Journal of Biological Chemistry*, vol. 281, no. 21, pp. 14907-14917.

Pamer, E. & Cresswell, P. 1998, "Mechanisms of MHC Class I - Restricted antigen processing", *Annual Review of Immunology*, vol. 16, pp. 323-358.

Parida, S., Oh, Y., Reid, S. M., Cox, S. J., Statham, R. J., Mahapatra, M., Anderson, J., Barnett, P. V., Charleston, B., & Paton, D. J. 2006, "Interferon-gamma production in vitro from whole blood of foot-and-mouth disease virus (FMDV) vaccinated and infected cattle after incubation with inactivated FMDV", *Vaccine*, vol. 24, no. 7, pp. 964-969.

Park J.Y., Moon J.S., Kim S.H., Kwon N.H., Ahn J.S., Davis W.C., & Davies C.J. 2004, "Characterization of lymphocyte subpopulations and major histocompatibility complex haplotypes of mastitis-resistant and susceptible cows**.**", *journal of veterinary science*, vol. 5, no. 1, pp. 29-39.

Paton, D. J., Sumption, K. J., & Charleston, B. 2009, "Options for control of foot-and-mouth disease: knowledge, capability and policy", *Philosophical Transactions of the Royal Society B-Biological Sciences*, vol. 364, no. 1530, pp. 2657-2667.

Patterson, H. & Thompson, R. 1971, "Recovery of inter-block information when block sizes are unequal", *Biometrika*, vol. 58, no. 3, p. 545-&.

Penn, D. J., Damjanovich, K., & Potts, W. K. 2002, "MHC heterozygosity confers a selective advantage against multiple-strain infections", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 17, pp. 11260-11264.

Pfaffl, M. W. 2001, "A new mathematical model for relative quantification in real-time RT-PCR", *Nucleic Acids Research*, vol. 29, no. 9.

Pieters, J. 1997, "MHC Class II restricted antigen presentation", *Current Opinion in Immunology*, vol. 9, no. 1, pp. 89-96.

Pipalia, D. L., Joshi, C. G., Rank, D. N., Brahmkshtri, B. P., & Solanki, J. V. 2004, "PCR-SSCP typing of MHC in cattle and buffaloes", *Indian Journal of Animal Sciences*, vol. 74, no. 6, pp. 637-639.

Poland, G. A., Ovsyannikova, I. G., Jacobson, R. M., & Smith, D. I. 2007, "Heterogeneity in vaccine immune response: The role of immunogenetics and the

emerging field of vaccinomics", *Clinical Pharmacology & Therapeutics*, vol. 82, no. 6, pp. 653-664.

Poland, G. A., Ovsyannikova, N. G., & Jacobson, R. M. 2008, "Vaccine immunogenetics: Bedside to bench to population", *Vaccine*, vol. 26, no. 49, pp. 6183-6188.

Rothbard, J. B. & Taylor, W. R. 1988, "A sequence pattern common to T-cell epitopes", *Embo Journal*, vol. 7, no. 1, pp. 93-100.

Rozen, S. & Skaletsky, H. J. Primer3 on the WWW for general users and for biologist programmers. Krawetz, S and Misener, S. Bioinformatics Methods and Protocols: Methods in Molecular Biology , 365-386. 2000. Humana Press, Totowa.

Rupp, R. & Boichard, D. 2003, "Genetics of resistance to mastitis in dairy cattle", *Veterinary Research*, vol. 34, no. 5, pp. 671-688.

Rupp, R., Hernandez, A., & Mallard, B. A. 2007, "Association of bovine leukocyte antigen (BoLA) DRB3.2 with immune response, mastitis, and production and type traits in Canadian Holsteins", *Journal of Dairy Science*, vol. 90, no. 2, pp. 1029-1038.

Russell, G. C., Davies, C. J., Andersson, L., Ellis, S. A., Hensen, E. J., Lewin, H. A., Mikko, S., MuggliCockett, N. E., & vanderPoel, J. J. 1997a, "BoLA Class II nucleotide sequences, 1996: Report of the ISAG BoLA Nomenclature Committee", *Animal Genetics*, vol. 28, no. 3, pp. 169-180.

Russell, G. C., Gallagher, A., Craigmile, S., & Glass, E. J. 1997b, "Characterization of cattle cDNA sequences from two DQA loci", *Immunogenetics*, vol. 45, no. 6, pp. 455-458.

Sadir, A. M., Zamorano, P. I., Romera, A., Wigdorovitz, A., Smitsaart, E., Marangunich, L., Schiappacassi, C., & Borca, M. V. 1999, "Improvement of the immune response to foot and mouth disease virus vaccine in calves by using Avridine as adjuvant", *Veterinary Immunology and Immunopathology*, vol. 69, no. 1, pp. 11-22.

Schrijver, R. S., Hensen, E. J., Langedijk, J. P. M., Daus, F., Middel, W. G. J., Kramps, J. A., & van Oirschot, J. T. 1997, "Antibody responses against epitopes on the F protein of bovine respiratory syncytial virus differ in infected or vaccinated cattle", *Archives of Virology*, vol. 142, no. 11, pp. 2195-2210.

Sette, A. & Fikes, J. 2003, "Epitope-based vaccines: an update on epitope identification, vaccine design and delivery", *Current Opinion in Immunology*, vol. 15, no. 4, pp. 461-470.

Shams, H. 2005, "Recent developments in veterinary vaccinology", *Veterinary Journal*, vol. 170, no. 3, pp. 289-299.

Sharif, S., Mallard, B. A., & Sargeant, J. M. 2000, "Presence of glutamine at position 74 of pocket 4 in the BoLA-DR antigen binding groove is associated with occurrence of

clinical mastitis caused by Staphylococcus species", *Veterinary Immunology and Immunopathology*, vol. 76, no. 3-4, pp. 231-238.

Sharif, S., Mallard, B. A., & Wilkie, B. N. 2003, "Characterization of naturally processed and presented peptides associated with bovine major histocompatibility complex (BoLA) Class II DR molecules", *Animal Genetics*, vol. 34, no. 2, pp. 116-123.

Sharif, S., Mallard, B. A., Wilkie, B. N., Sargeant, J. M., Scott, H. M., Dekkers, J. C. M., & Leslie, K. E. 1999, "Associations of the bovine major histocompatibility complex DRB3 (BoLA-DRB3) with production traits in Canadian dairy cattle", *Animal Genetics*, vol. 30, no. 2, pp. 157-160.

Siegrist, C. A. 2003, "Mechanisms by which maternal antibodies influence infant vaccine responses: review of hypotheses and definition of main determinants", *Vaccine*, vol. 21, no. 24, pp. 3406-3412.

Sigurdardottir, S., Borsch, C., Gustafsson, K., & Andersson, L. 1992, "Gene duplications and sequence polymorphism of bovine Class-II *DQB* genes", *Immunogenetics*, vol. 35, no. 3, pp. 205-213.

Silverstein, A. M. 2009, in *A history of immunology*, 2nd edn, Elsevier, pp. 231-259.

Sitte, K., Brinkworth, R., East, I. J., & Jazwinska, E. C. 2002, "A single amino acid deletion in the antigen binding site of BoLA-DRB3 is predicted to affect peptide binding", *Veterinary Immunology and Immunopathology*, vol. 85, no. 3-4, pp. 129-135.

Snowder, G. D., Van Vleck, L. D., Cundiff, L. V., & Bennett, G. L. 2006, "Bovine respiratory disease in feedlot cattle: Environmental, genetic, and economic factors", *Journal of Animal Science*, vol. 84, no. 8, pp. 1999-2008.

Sobrino, F., Saiz, M., Jimenez-Clavero, M. A., Nunez, J. I., & Rosas, M. F. 2001, "Foot-and-mouth disease virus: a long known virus, but a current threat", *Veterinary Research*, vol. 32, no. 1, pp. 1-30.

Sordillo, L. M. & Streicher, K. L. 2002, "Mammary gland immunity and mastitis susceptibility", *Journal of Mammary Gland Biology and Neoplasia*, vol. 7, no. 2, pp. 135-146.

Steimle, V., Siegrist, C. A., Mottet, A., Lisowskagrospierre, B., & Mach, B. 1994, "Regulation of MHC Class-II expression by interferon-gamma mediated by the transactivator cene CIITA", *Science*, vol. 265, no. 5168, pp. 106-109.

Stern, L. J., Brown, J. H., Jardetzky, T. S., Gorga, J. C., Urban, R. G., Strominger, J. L., & Wiley, D. C. 1994, "Crystal-structure of the human Class-II MHC protein HLA-DR1 complexed with an influenza-virus peptide", *Nature*, vol. 368, no. 6468, pp. 215-221.

Stott, E. J., Thomas, L. H., Collins, A. P., Crouch, S., Jebbett, J., Smith, G. S., Luther, P. D., & Caswell, R. 1980, "A survey of virus-infections of the respiratory-tract of cattle and their association with disease", *Journal of Hygiene*, vol. 85, no. 2, pp. 257-270.

Summerfield, A., Guzylack-Piriou, L., Harwood, L., & Mccullough, K. C. 2009, "Innate immune responses against foot-and-mouth disease virus: Current understanding and future directions", *Veterinary Immunology and Immunopathology*, vol. 128, no. 1-3, pp. 205-210.

Sutmoller, P., Barteling, S. S., Olascoaga, R. C., & Sumption, K. J. 2003, "Control and eradication of foot-and-mouth disease", *Virus Research*, vol. 91, no. 1, pp. 101-144.

Taboga, O., Tami, C., Carrillo, E., Nunez, J. I., Rodriguez, A., Saiz, J. C., Blanco, E., Valero, M. L., Roig, X., Camarero, J. A., Andreu, D., Mateu, M. G., Giralt, E., Domingo, E., Sobrino, F., & Palma, E. L. 1997, "A large-scale evaluation of peptide vaccines against foot-and-mouth disease: Lack of solid protection in cattle and isolation of escape mutants", *Journal of Virology*, vol. 71, no. 4, pp. 2606-2614.

Takeshima, S., Matsumoto, Y., Chen, J., Yoshida, T., Mukoyama, H., & Aida, Y. 2008, "Evidence for cattle major histocompatibility complex (BoLA) Class II DQA1 gene heterozygote advantage against clinical mastitis caused by Streptococci and Escherichia species", *Tissue Antigens*, vol. 72, no. 6, pp. 525-531.

Takeshima, S., Miki, A., Kado, M., & Aida, Y. 2007, "Establishment of a sequence-based typing system for BoLA-DQA1 exon 2", *Tissue Antigens*, vol. 69, no. 2, pp. 189-199.

Takeshima, S., Sarai, Y., Saitou, N., & Aida, Y. 2009, "MHC Class II DR classification based in antigen-binding groove natural selection", *Biochemical and Biophysical Research Communications*, vol. 385, no. 2, pp. 137-142.

Takeshima, S. N. & Aida, Y. 2006, "Structure, function and disease susceptibility of the bovine major histocompatibility complex", *Animal Science Journal*, vol. 77, no. 2, pp. 138-150.

Talbot, B. G. & Lacasse, P. 2005, "Progress in the development of mastitis vaccines", *Livestock Production Science*, vol. 98, no. 1-2, pp. 101-113.

Taylor, G., Stott, E. J., Furze, J., Ford, J., & Sopp, P. 1992, "Protective epitopes on the fusion protein of respiratory syncytial virus recognized by murine and bovine monoclonal-antibodies", *Journal of General Virology*, vol. 73, pp. 2217-2223.

Taylor, G., Thomas, L. H., Wyld, S. G., Furze, J., Sopp, P., & Howard, C. J. 1995, "Role of T-lymphocyte subsets in recovery from respiratory syncytial virus-infection in calves", *Journal of Virology*, vol. 69, no. 11, pp. 6658-6664.

The Bovine Genome Sequencing and Analysis Consortium, Elsik, C. G., Tellam, R. L., & Worley, K. C. 2009, "The Genome Sequence of Taurine Cattle: A Window to Ruminant Biology and Evolution", *Science*, vol. 324, no. 5926, pp. 522-528.

The MHC sequencing consortium 1999, "Complete sequence and gene map of a human major histocompatibility complex", *Nature*, vol. 401, no. 6756, pp. 921-923.

The Royal Society

2002

Internet Communication

http://royalsociety.org/General_WF.aspx?pageid=7836&terms=foot+and+mouth

Thompson, D., Muriel, P., Russell, D., Osborne, P., Bromley, A., Rowland, M., Creigh-Tyte, S., & Brown, C. 2002, "Economic costs of the foot and mouth disease outbreak in the United Kingdom in 2001", *Revue Scientifique et Technique de l Office International des Epizooties*, vol. 21, no. 3, pp. 675-687.

Thursz, M. R., Thomas, H. C., Greenwood, B. M., & Hill, A. V. S. 1997, "Heterozygote advantage for HLA Class-II type in hepatitis B virus infection", *Nature Genetics*, vol. 17, no. 1, pp. 11-12.

Traul, D. L., Bhushan, B., Eldridge, J. A., Crawford, T. B., Li, H., & Davies, C. J. 2005, "Characterization of Bison bison major histocompatibility complex Class IIa haplotypes", *Immunogenetics*, vol. 57, no. 11, pp. 845-854.

Trowsdale, J. 2001, "Genetic and functional relationships between MHC and NK receptor genes", *Immunity*, vol. 15, no. 3, pp. 363-374.

Udina, I. G., Karamysheva, E. E., Turkova, S. O., Orlova, A. R., & Sulimova, G. E. 2003, "Genetic mechanisms of resistance and susceptibility to leukemia in Ayrshire and Black Pied cattle breeds determined by allelic distribution of gene BoLA-DRB3", *Russian Journal of Genetics*, vol. 39, no. 3, pp. 306-317.

Valarcher, J. F. & Taylor, G. 2007, "Bovine respiratory syncytial virus infection", *Veterinary Research*, vol. 38, no. 2, pp. 153-180.

Valitutti, S., Muller, S., Cella, M., Padovan, E., & Lanzavecchia, A. 1995, "Serial triggering of many T-cell receptors by a few peptide-MHC complexes", *Nature*, vol. 375, no. 6527, pp. 148-151.

Van Eijk, M. J. T., Stewarthaynes, J. A., & Lewin, H. A. 1992, "Extensive polymorphism of the BoLA-DRB3 gene distinguished by PCR-RFLP", *Animal Genetics*, vol. 23, no. 6, pp. 483-496.

Van Lierop, M. J. C., Nilsson, P. R., Wagenaar, J. P. A., Vannoort, J. M., Campbell, J. D. M., Glass, E. J., Joosten, I., & Hensen, E. J. 1995, "The influence of MHC polymorphism on the selection of T-cell determinants of FMDV in cattle", *Immunology*, vol. 84, no. 1, pp. 79-85.

Van Oirschot, J. T. 1999, "Diva vaccines that reduce virus transmission", *Journal of Biotechnology*, vol. 73, no. 2-3, pp. 195-205.

Vangeel, I., Antonis, A. F. G., Fluess, M., Peters, A. R., & Harmeyer, S. S. 2005, "Efficacy of a modified live intranasal bovine respiratory syncytial virus vaccine in

three-week-old calves experimentally challenged with BRSV", *Cattle Practice*, vol. 13, pp. 263-271.

Vidan-Jeras, B., Brinovec, V., Jurca, B., Stezinar, S. n. L., Jeras, M., & Bohinjec, M. 2000, "The contribution of HLA-Class II antigens in humoral non-response and delayed response to HBsAg vaccination", *Pflugers Archiv European Journal of Physiology*, vol. 440, no. 7, p. r188-r189.

Vogt, A. B., Kropshofer, H., & Hammerling, G. J. 1997, "How HLA-DM affects the peptide repertoire bound to HLA-DR molecules", *Human Immunology*, vol. 54, no. 2, pp. 170-179.

Vordermeier, M., Whelan, A. O., & Hewinson, R. G. 2003, "Recognition of mycobacterial epitopes by T cells across mammalian species and use of a program that predicts human HLA-DR binding peptides to predict bovine epitopes", *Infection and Immunity*, vol. 71, no. 4, pp. 1980-1987.

Wang, C. Y., Chang, T. Y., Walfield, A. M., Ye, J., Shen, M., Chen, S. P., Li, M. C., Lin, Y. L., Jong, M. H., Yang, P. C., Chyr, N., Kramer, E., & Brown, F. 2002, "Effective synthetic peptide vaccine for foot-and-mouth disease in swine", *Vaccine*, vol. 20, no. 19-20, pp. 2603-2610.

Wang, C. Y. J. & Smith, B. F. 2007, "Development of quantitative polymerase chain reaction assays for allelic discrimination of gangliosidoses in cats", *American Journal of Veterinary Research*, vol. 68, no. 3, pp. 231-235.

Wang, K., Sun, D., Xu, R., & Zhang, Y. 2005, "Identification of 19 new BoLA-DQB alleles", *Animal Genetics*, vol. 36, no. 2, pp. 166-167.

Weenink, S. M. & Gautam, A. M. 1997, "Antigen presentation by MHC Class II molecules", *Immunology and Cell Biology*, vol. 75, no. 1, pp. 69-81.

Wiesmuller, K. H., Fleckenstein, B., & Jung, G. 2001, "Peptide vaccines and peptide libraries", *Biological Chemistry*, vol. 382, no. 4, pp. 571-579.

Wilkie, B. N. & Mallard, B. A. 1999, "Genetic effects on vaccination", *Advances in Veterinary Medicine, Vol 41*, vol. 41, pp. 39-51.

Wilson, D. J., Gonzalez, R. N., & Das, H. H. 1997, "Bovine mastitis pathogens in New York and Pennsylvania: Prevalence and effects on somatic cell count and milk production", *Journal of Dairy Science*, vol. 80, no. 10, pp. 2592-2598.

Winkler, M. T. C., Doster, A., & Jones, C. 1999, "Bovine herpesvirus 1 can infect CD4(+) T lymphocytes and induce programmed cell death during acute infection of cattle", *Journal of Virology*, vol. 73, no. 10, pp. 8657-8668.

Wiseman, R. W., Karl, J. A., Bimber, B. N., O'Leary, C. E., Lank, S. M., Tuscher, J. J., Detmer, A. M., Bouffard, P., Levenkova, N., Turcotte, C. L., Szekeres, E., Wright, C., Harkins, T., & O'Connor, D. H. 2009, "Major histocompatibility complex genotyping

with massively parallel pyrosequencing", *Nature Medicine*, vol. 15, no. 11, p. 1322-U123.

Woelfing, B., Traulsen, A., Milinski, M., & Boehm, T. 2009, "Does intra-individual major histocompatibility complex diversity keep a golden mean?", *Philosophical Transactions of the Royal Society B-Biological Sciences*, vol. 364, no. 1513, pp. 117-128.

Woodbine, K., Medley, G., Moore, S., Ramirez-Villaescusa, A., Mason, S., & Green, L. 2009, "A four year longitudinal sero-epidemiological study of bovine herpesvirus type-1 (BHV-1) in adult cattle in 107 unvaccinated herds in south west England", *BMC Veterinary Research*, vol. 5, no. 1, pp. 5-18.

Xu, A. L., Park, C., & Lewin, H. A. 1994, "Both DQB genes are expressed in BoLA haplotypes carrying a duplicated DQ region", *Immunogenetics*, vol. 39, no. 5, pp. 316-321.

Xu, A. L., Vaneijk, M. J. T., Park, C., & Lewin, H. A. 1993, "Polymorphism in BoLA-DRB3 exon-2 correlates with resistance to persistent lymphocytosis caused by Bovine Leukemia-Virus", *Journal of Immunology*, vol. 151, no. 12, pp. 6977-6985.

Yates, W. D. 1982, "A review of infectious bovine rhinotracheitis, shipping fever pneumonia and viral-bacterial synergism in respiratory disease of cattle", *Canadian Journal of Comparative Medicine*, vol. 46, no. 3, pp. 225-263.

Yoshida, T., Mukoyama, H., Furuta, H., Kondo, Y., Takeshima, S., Aida, Y., Kosugiyama, M., & Tomogane, H. 2009, "Association of the amino acid motifs of BoLA-DRB3 alleles with mastitis pathogens in Japanese Holstein cows", *Animal Science Journal*, vol. 80, no. 5, pp. 510-519.

Young, F. J. 2002, *Innate and Acquired Immune Response in Crossbred Cattle,* PhD, University of Glasgow.

Young, F. J., Woolliams, J. A., Williams, J. L., Glass, E. J., O'Neill, R. G., & Fitzpatrick, J. L. 2005, "In vitro peripheral blood mononuclear cell proliferation in a crossbred cattle population", *Journal of Dairy Science*, vol. 88, no. 7, pp. 2643-2651.

Zatechka, D. S., Hegde, N. R., Hariharan, K., & Srikumaran, S. 1999, "Identification of murine cytotoxic T-lymphocyte epitopes of bovine herpesvirus 1", *Vaccine*, vol. 17, no. 7-8, pp. 686-694.

Zavala-Ruiz, Z., Strug, I., Anderson, M. W., Gorski, J., & Stern, L. J. 2004, "A polymorphic pocket at the P10 position contributes to peptide binding specificity in Class II MHC proteins", *Chemistry & Biology*, vol. 11, no. 10, pp. 1395-1402.

Zerva, L., Cizman, B., Mehra, N. K., Alahari, S. K., Murali, R., Zmijewski, C. M., Kamoun, M., & Monos, D. S. 1996, "Arginine at positions 13 or 70-71 in pocket 4 of HLA-DRB1 alleles is associated with susceptibility to tuberculoid leprosy", *Journal of Experimental Medicine*, vol. 183, no. 3, pp. 829-836.

Zhang, Z. D., Hutching, G., Kitching, P., & Alexandersen, S. 2002, "The effects of gamma interferon on replication of foot-and-mouth disease virus in persistently infected bovine cells", *Archives of Virology*, vol. 147, no. 11, pp. 2157-2167.

Zinkernagel, R. M. & Doherty, P. C. 1974, "Restriction of in-vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system", *Nature*, vol. 248, no. 5450, pp. 701-702.

Zygraich "Vaccination against RS virus - a five year experience with Rispoval", Proceedings of the 12th World Congress on diseases of cattle edn, pp. 189-195.

Appendix A.1

Human mRNA MHC gene list and RefSeq IDs

Human MHC genes	Human mRNA RefSeq ID
HLA-F	NM_018950
HLA-G	NM_002127
HLA-A	NM_002116
ZNRD1	NM_014596
PPP1R11	NM_170781
TRIM31	NM_052816
TRIM10	NM_006778
TRIM15	NM_052812
TRIM26	NM_003449
HLA-E	NM_005516
GNL1	NM_005275
PRR3	NM_025263
ABCF1	NM_001090
MRPS18B	NM_014046
DHX16	NM_003587
MDC1	NM_014641
TUBB	NM_001069
IER3	NM_003897
DDR1	NM_001954
C6orf15	NM_014070
CDSN	NM_001264
PSORS1C2	NM_014069
C6orf18	NM_019052
TCF19	NM_007109
HLA-C	NM_002117
HLA-B	NM_005514

MICB	NM_005931
HLA-DRA	NM_019111
HLA-DRB3	NM_022555
HLA-DRB1	NM_002124
HLA-DQA1	NM_002122
HLA-DQB1	NM_002123
HLA-DOA	NM_002119
HLA-DPA1	NM_033554
BAT1	NM_080598
ATP6V1G2	NM_130463
NFKBIL1	NM_005007
LTA	NM_000595
TNF	NM_000594
LTB	NM_009588
NCR3	NM_147130
AIF1	NM_004847
BAT2	NM_080686
BAT3	NM_080703
APOM	NM_019101
C6orf47	NM_021184
BAT4	NM_033177
CSNK2B	NM_001320
LY6G5B	NM_021221
LY6G5C	NM_025262
BAT5	NM_021160
LY6G6E	NM_024123
C6orf25	NM_138277
DDAH2	NM_013974
CLIC1	NM_001288
MSH5	NM_025259
C6orf27	NM_025258
VARS2	NM_006295

LSM2	NM_021177
HSPA1L	NM_005527
HSPA1A	NM_005345
HSPA1B	NM_005346
NEU1	NM_000434
C6orf29	NM_032794
BAT8	NM_025256
ZBTB12	NM_181842
C2	NM_000063
BF	NM_001710
RDBP	NM_002904
SKIV2L	NM_006929
DOM3Z	NM_032419
STK19	NM_032454
C4B	NM_000592
CYP21A2	NM_000500
TNXB	NM_032470

Bovine mRNA MHC gene list and RefSeq IDs

Bovine MHC genes	Bovine mRNA RefSeq ID
BoLA-A	NM_001040532
BoLA DRB3	NM_001012680.2
BoLA DRA	NM_001012677.1

Appendix B.1

Nucleotide code	Base
A	Adenine
C	Cytosine
G	Guanine
T	Thymidine
R	A or G
Y	C or T
S	G or C
W	A or T
K	G or T
M	A or C

IUPAC nomenclature for nucleotide bases.

DRB3 allele allocations for RoBoGen animals

Animal ID	Sire ID	Allele 1	Allele2
1	R11	0501	1601
2	R21	1101	0701
3	R21	1101	1601
4	H26	2707	2002
5	R02	2707	2707
6	R11	1601	2707
7	R05	0201	1101
8	R21	1101	0701
9	R11	1101	2707
10	R05	0201	1501
11	R19	0701	0901
12	R01	0601	2707
13	R11	0501	1101
14	R21	1101	1101
15	R21	0101	1101
16	R11	2707	1101

17	R21	0201	1101
18	R01	0201	1101
19	R19	0901	1601
20	R19	0901	1601
21	R15	2707	1002
22	H26	2707	1101
23	R11	0501	2703
24	R21	0201	1101
25	R21	0201	1001
26	R01	0101	1101
27	H25	1601	2707
28	R01	0601	0201
29	R21	0501	1101
30	R05	0201	1601
31	R02	0601	2707
32	R01	0601	2707
33	R15	1201	2707
34	H27	2707	2707
35	R11	1601	2707
36	H26	0201	0101
37	R15	2707	0701
38	R19	1601	2703
39	H27	0901	2707
40	H25	1601	2707
41	R19	0901	1101
42	H26	0201	2707
43	H26	1701	2707
44	R02	0601	2707
45	H25	1601	2707
46	H27	0501	1101
47	H27	0701	1101
48	R11	0501	1101
49	H27	1101	0701
50	H25	0501	1101
51	H25	1601	2707
52	R19	0101	0901

53	R19	0901	0101
54	R11	0701	2707
55	R02	0601	0901
56	R01	0901	1101
57	R01	1101	2707
58	R02	0901	2707
59	R02	0902	2707
60	R02	2707	2707
61	R02	0201	2707
62	R01	1101	2002
63	R02	0601	2703
64	R19	0901	1101
65	R19	1601	1701
66	R19	1601	0201
67	R11	0902	2707
68	R02	0601	0901
69	R15	1201	1601
70	R01	0601	2002
71	R21	1101	2707
72	R15	2707	0101
73	R01	0601	2002
74	R02	2707	0601
75	R02	0601	0902
76	R19	1601	2707
77	R02	0601	2703
78	R15	2707	1201
79	R11	0501	1201
80	R02	0601	0901
81	R05	1601	2703
82	R02	0901	2707
83	R11	0501	0501
84	R02	2707	0901
85	R02	0601	2707
86	R02	1601	2707
87	R05	0201	0201
88	R05	0201	0201

89	R05	0201	0101
90	R21	1101	0101
91	R15	1201	0201
92	R19	0901	0901
93	R01	1101	1101
94	R01	1101	14011
95	R02	0601	0902
96	R02	0902	2707
97	R01	1101	1101
98	R11	0501	1101
99	R19	0901	0201
100	H26	1601	2707
101	R02	0601	2707
102	H26	0201	1201
103	R21	1001	1001
104	R01	0601	1201
105	H26	2707	2707
106	H26	2707	2707
107	R11	2707	0902
108	R02	0601	0902
109	R05	0201	1201
110	H27	2707	0701
111	R05	1601	1101
112	R05	1601	2707
113	R21	1001	0201
114	R21	1001	1201
115	H27	1101	0101
116	R19	0901	0701
117	R02	0601	0902
118	R21	1001	0902
119	R05	1601	0101
120	R19	1601	3201
121	R15	1201	1601
122	H27	2707	2707
123	R19	1601	2707
124	R11	0501	1201

125	R05	1601	1601
126	R19	1601	1601
127	R21	1001	1201
128	R15	0101	0101
129	R19	0901	1601
130	R21	2707	1001
131	R19	0901	0101
132	R19	1601	1601
133	R19	0901	0101
134	R19	1601	0701
135	R02	2707	2703
136	R19	1601	1101
137	R19	1601	2707
138	R19	0901	0101
139	R02	2707	1201
140	R12	1101	1201
141	R15	2707	2707
142	R19	1601	0201
143	R19	0901	1101
144	R19	0901	0701
145	R11	0501	2707
146	R19	1601	2707
147	H26	0201	1001
148	R11	2707	0101
149	H25	1601	2002
150	H25	1101	2707
151	R01	1101	2707
152	H25	1601	2707
153	R11	2707	2707
154	R21	1101	0901
155	R01	0601	2707
156	R12	1101	1601
157	R05	1601	1001
158	R11	2707	0901
159	R02	2707	0601
160	R02	2707	0601

161	R11	0501	1101
162	R15	1201	1601
163	R21	1101	1101
164	R15	1201	1101
165	R11	2707	0101
166	R01	1101	0201
167	H27	2707	0101
168	R19	1601	1101
169	H26	0201	2707
170	R05	1601	1102
171	R15	1201	1101
172	R21	1101	2707
173	R15	2707	0201
174	R01	1101	2707
175	R19	0901	1101
176	R01	0601	1101
177	R05	0201	0101
178	R02	0101	2707
179	R21	0701	1001
180	R11	2707	1101
181	R12	2707	1101
182	R02	0601	2707
183	H26	1101	2707
184	R12	2707	1601
185	R02	2707	1201
186	R19	1601	0901
187	R05	0201	1101
188	R05	0201	0201
189	R02	0601	1101
190	R19	1601	1101
191	R21	1001	2703
192	H27	2707	0701
193	R21	1001	1801
194	R01	0601	0501
195	R02	0601	1701
196	H27	1101	2707

197	R02	2707	0101
198	R19	1601	2002
199	H27	2707	1601
200	H27	2707	0701
201	R02	0601	1501
202	H27	1101	1801
203	H147	2707	1101
204	H147	2707	1101
205	H25	1101	0902
206	R01	1101	0101
207	R21	1001	0501
208	R21	1101	0101
209	R21	1001	1801
210	R12	1101	0101
211	R02	2707	1201
212	H25	1601	1601
213	R12	1101	2703
214	H27	2707	0101
215	R05	0201	1501
216	R15	2707	1601
217	R01	0601	0701
218	H26	0201	0101
219	R15	1201	1601
220	R02	1101	2707
221	H26	1001	2707
222	R21	0601	1101
223	R15	2707	2707
224	H25	1101	0201
225	R02	2707	1101
226	R21	2707	1101
227	R15	1201	2707
228	R11	1601	2707
229	R21	1001	1101
230	R12	2707	1102
231	R15	1201	1101
232	R19	0901	0101

233	R19	0901	1101
234	H25	1601	2707
235	H26	2707	1201
236	R19	1601	0902
237	R21	1101	0101
238	H26	2707	2707
239	H25	1601	0501
240	H25	1601	2707
241	H27	2707	2707
242	H27	1101	1202
243	H26	0201	0701
244	R19	1601	2801
245	R12	2707	0501
246	R19	1601	1801
247	R05	1601	1001
248	R15	2707	0902
249	R01	1101	0101
250	R11	2707	0101
251	R05	1601	0501
252	R21	1001	1501
253	R19	0901	3201
254	R12	1101	1701
255	R05	1601	0601
256	R19	1601	1101
257	R15	2707	1001
258	R19	1601	2707
259	H27	1101	0901
260	R21	1101	0701
261	H147	1701	1201
262	R11	0501	0101
263	R21	1101	0101
264	R21	1101	0101
265	R12	2707	1801
266	R12	2707	0902
267	R11	0501	1101
268	R15	2707	0101

269	R01	1101	1001
270	R19	0901	1101
271	H26	2707	0701
272	R19	0901	0201
273	R11	2707	2707
274	R15	2707	0701
275	R19	0901	0101
276	R15	2707	1501
277	H26	2707	2707
278	H147	2707	0201
279	R02	2707	0101
280	R12	2707	0601
281	R05	1601	0101
282	R19	1601	2707
283	R19	0901	1001
284	R19	0901	2707
285	R19	0901	1701
286	R19	1601	1101
287	R19	0901	0101
288	R01	1101	0201
289	R19	0901	0901
290	R19	1601	2707
291	R01	0601	0101
292	R19	0901	0101
293	R19	0901	0101
294	R21	1001	1601
295	R21	1101	1601
296	H27	2707	0501
297	R21	1101	1101
298	R21	1101	0701
299	R21	1001	1101
300	R11	2707	1101
301	R11	1601	0501
302	R05	0201	1001
303	R05	0201	1001
304	R12	2707	2703

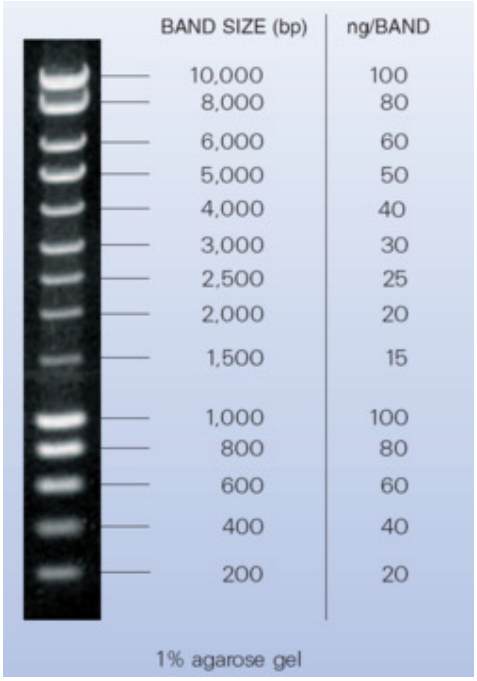
305	R02	2707	2002
306	R05	0201	1101
307	R05	0201	1501
308	R05	1601	1201
309	RO5	1601	1201
310	RO5	1001	1601
311	R21	1001	0201
312	H25	1601	2707
313	R12	1101	0101
314	R02	2707	2707
315	R02	0601	0601
316	R15	2707	0101
317	R02	2707	1101
318	R21	1101	1801
319	R01	1101	0201
320	R02	2707	2707
321	H26	0201	2707
322	CH 108	2707	2707
323	R21	1101	1101
324	R05	1601	1601
325	R21	1001	1101
326	R21	1101	0801
327	R15	2707	2707
328	R01	0601	14011
329	R02	2707	0902
330	R21	1001	2707
331	R19	1601	3201
332	H147	1707	0101
333	R01	1101	0201
334	H25	1601	1001
335	R19	1601	0101
336	H147	2707	1101
337	R05	1601	1601
338	H25	1101	1201
339	R21	1001	1001
340	H25	1601	2707

341	R15	1201	1001
342	R01	0601	0601
343	H147	2707	0601
344	R01	1101	1001
345	R01	1101	1201
346	R11	2707	1101
347	R15	1201	1101
348	R15	1201	1101
349	R19	1601	1701
350	R05	0201	0501
351	H27	2707	0101
352	R12	1101	0902
353	R19	1601	2707
354	R19	0901	0101
355	R19	0901	0101
356	R21	1001	1201
357	R19	0901	1101
358	R12	2707	1201
359	R02	0601	1601
360	R01	0601	0601
361	R15	1201	1101
362	R15	1201	1101
363	H147	1701	2707
364	R19	1601	0101
365	R15	2707	1101
366	R11	0501	1201
367	R19	0101	1601
368	R12	1101	1201
369	H26	2707	2707
370	R19	1601	0101
371	R12	0201	1101
372	R19	0901	1101
373	R19	1601	2002
374	R15	1201	1101
375	R19	1601	1601
376	R02	0601	0601

377	R19	1601	0201
378	R11	0501	1101
379	R19	0901	0101
380	R19	1601	0101
381	R19	0901	0101
382	R01	1101	1501
383	R15	1201	1101
384	R19	0901	0601
385	R15	2707	1101
386	R11	2707	1101
387	R11	0501	0101
388	R15	2707	1601
389	R02	2707	2002
390	R15	1201	0201
391	R15	2707	0601
392	R05	0201	1101
393	R21	0501	1101
394	R11	0501	1101
395	R15	1201	0902
396	R15	2707	1601
397	R19	1601	0201
398	R15	2707	1101
399	R11	0501	0601
400	R15	1201	1201
401	R19	1601	2707
402	R15	2707	0902
403	R19	0901	2707
404	R19	0901	1101
405	R19	1601	1101
406	R19	0901	1201
407	R19	1601	1701
408	R19	0901	0101
409	R19	1601	1201

Appendix B.2

Hyperladder I.



Hyperladder I (Bioline, UK) used as molecular weight ladder in all agarose gel electrophoresis images.

Appendix C.1

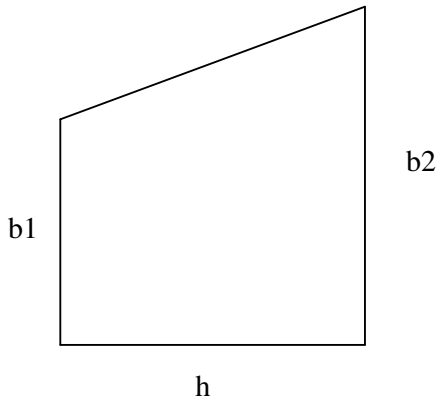
Trapezoid rule

Allows estimate of area under a line. The formula is used in geometry to calculate the area of a trapezoid, height (h), base length (b1) and base length (b2). By subdividing the area under a line the total area can be calculated.

Area of trapezoid is:

$$\mathbf{1/2h.(b1+b2)}$$

Where:



Appendix C.2

Cell proliferation assay for response to FMDV15 and ConA (Chapter 3)

Whole blood in heparin (150 µl) was diluted with 750 µl RPMI 1640 (Sigma, UK) supplemented with 25 mM HEPES, 2 mM glutamine (Invitrogen, UK), 10% foetal calf serum (Perbio, EU origin), 5 x 10^{-5} M 2-mercaptoethanol (Invitrogen). For each animal, seven cultures were prepared: a medium only negative control, three final concentrations of concanavalin A (ConA) (0.25, 2.5 and 10 µg/ml (Sigma, UK) as positive controls and three final concentrations of FMDV15 peptide (0.1, 1 and 2.0 µg/ml). Quadruplicate cultures in 96-well tissue culture grade flat plates (Nunc, UK) were incubated at 37^{o}C with 5% CO_2 for 6 days. For the last 6 hours the cells were labelled with 0.037 MBq ^{3}H-Thymidine per well (GE Healthcare, UK) and uptake assessed by liquid scintillation counting using a 1450 Microbeta (Wallac, now PerkinElmer). Results were expressed as counts per minute (cpm) of ^{3}H-Thymidine incorporation (mean of quadruplicates).

IFN-γ assay for response to FMDV15 and ConA (Chapter 3)

1ml of whole heparinised blood was diluted with 1ml RPMI supplemented as described above for the cell proliferation assay, and was added to each well of a 24-well plate (Nunc, UK). Each animal had three samples: medium alone as the negative control, 2.5 ug/ml ConA as the positive control, and 1ug/ml FMDV15 (both final concentrations).

Samples were incubated for 24 hours at 37^{o}C and 5% CO_2. After incubation, samples were mixed with a pipette and transferred to a centrifuge tube. Tubes were spun at 750 g for 10 minutes and supernatants harvested and frozen at -20^{o}C until use in the EASIA. All assay tests were done in duplicate. The EASIA kit is a 2 step enzyme immunometric assay designed to measure bovine IFN-γ, and consists of a capture monoclonal antibody to bovine IFN-γ fixed to the well, and an anti-bovine IFN-γ horse radish peroxidise (HRP) conjugate. Firstly, 100 µl of the ovine rIFN-γ standards (4000, 3000, 2000, 1000, 500, 100 pg/ml diluted in RPMI supplemented as described above), control and test samples were added to 96-well strips together with 50µl of "Incubation Buffer" (Biosource kit) and incubated at room temperature for 60 minutes on a shaker, before being washed 3 times with 400 µl of "Working Washing Solution". 100 µl of appropriately diluted conjugate was added to each well, and again incubated at room temperature for 60 minutes, on a shaker. The plates were washed 3 further times and then 100 µl of TMB was added to each well and all samples were incubated at room temperature for 15 minutes on a shaker. Finally 200 µl of "Stop Solution" was added and the optical density of each well was read at 450 nm on a Victor^2 1420 Multilabel counter (Wallac). Optical density (OD) values were converted into concentrations using a standard curve derived from dilutions of ovine IFN-γ using a simple linear regression (Genstat, 9.0v, VSN International) and the results were expressed in pg/ml. The intra- and inter-plate replicates were high with r^2 values of at least 0.80.

FMDV IgG1 *DRB3* alleles and pockets

All results from REML model analysis for IgG1 response to FMDV15.

IgG1	*DRB3* alleles																	
WEEK	*0101*	*0201*	*0501*	*0601*	*0701*	*0901*	*0902*	*1001*	*1101*	*1201*	*1501*	*1601*	*1701*	*1801*	*2002*	*2703*	*2707*	*3201*
2	n.s.	n.s.	n.s.	n.s.	n.s.	0.031	n.s.	0.063	n.s.	n.s.	n.s.	0.035	n.s.	n.s.	n.s.	n.s.	<0.001	n.s.
4	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.004	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
8	n.s.	n.s.	n.s.	n.s.	n.s.	0.015	0.012	0.031	n.s.	n.s.	n.s.	0.023	n.s.	n.s.	n.s.	n.s.	0.041	n.s.
10	n.s.	n.s.	n.s.	n.s.	0.036	n.s.	n.s.	0.038	n.s.	n.s.	n.s.	0.007	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
AUC	n.s.	n.s.	n.s.	n.s.	0.034	<0.001	n.s.	0.02	n.s.	n.s.	n.s.	0.023	n.s.	n.s.	n.s.	n.s.	0.032	n.s.

p values from REML analysis of *DRB3* alleles and IgG1 response to FMDV15

Week IgG1	pocket 1	pocket 6	pocket 7						pocket 9					Pocket 10 pos 56
			28	30	47	61	67	71	9	37	57	60	61	
2	n.s.	0.020	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.048	0.042	n.s.	n.s.	n.s.
4	n.s.	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
8	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.022	n.s.	n.s.	n.s.	n.s.
10	0.011	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.017	n.s.	n.s.	n.s.	n.s

p values from REML analysis of binding pockets and IgG1 response to FMDV15

Pocket 4

	Pocket 4			
Weeks IgG1	13	70	71	74
2	n.s.	<0.001	n.s.	n.s.
4	n.s.	n.s.	n.s.	n.s.
8	n.s.	n.s.	n.s.	0.024
10	n.s.	n.s.	n.s.	n.s.
AUC	n.s.	n.s.	n.s.	n.s.

	position 13				position 70			position 71				position 74		
Weeks IgG1	G	S	K	R	E	R	Q	K	R	A	E	A	E	N
2	n.s.	n.s.	n.s.	0.029	<0.001	<0.001	n.s.	n.s.	0.022	n.s.	n.s.	n.s.	0.050	0.003
4	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.030
8	n.s.	n.s.	n.s.	n.s.	0.027	n.s.	n.s.	n.s.	0.092	n.s.	n.s.	n.s.	n.s.	0.012
10	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.094
AUC	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.001

p values from REML analysis of binding pocket 4 and IgG1 response to FMDV15

Appendix C.3

FMDV IgG2 *DRB3* alleles and pockets

All results from REML model analysis for IgG2 response to FMDV15.

IgG2	DRB3 alleles																	
WEEK	*0101*	*0201*	*0501*	*0601*	*0701*	*0901*	*0902*	*1001*	*1101*	*1201*	*1501*	*1601*	*1701*	*1801*	*2002*	*2703*	*2707*	*3201*
2	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.013	n.s.	n.s.	n.s.	<0.001	n.s.	n.s.	n.s.	n.s.	0.001	n.s.
4	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	<0.001	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
8	0.002	n.s.	n.s.	n.s.	0.026	n.s.	n.s.	0.005	n.s.	n.s.	n.s.	<0.001	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
10	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.012	n.s.	n.s.	n.s.	0.003	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
AUC	n.s.	n.s.	n.s.	n.s.	0.058	0.01	n.s.	0.047	n.s.	n.s.	n.s.	0.005	n.s.	n.s.	n.s.	n.s.	0.031	n.s.

: p values from the REML analysis of *DRB3* alleles with IgG2 response to FMDV15

Week IgG2	pocket 1	pocket 6	pocket 7						pocket 9					Pocket 10 pos 56
			28	30	47	61	67	71	9	37	57	60	61	
2	n.s.	<0.001	n.s.	0.029	n.s.	0.022	n.s.	n.s.	0.056	<0.001	<0.001	0.020	0.022	0.001
4	0.046	n.s.	0.036	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.036	n.s.	n.s.	0.018
8	0.031	n.s.	n.s.	n.s.	n.s.	0.009	n.s.	0.043	n.s.	n.s.	<0.001	0.083	0.011	<0.001
10	n.s.	n.s.	n.s.	n.s.	n.s.	0.041	n.s.	n.s.	n.s.	n.s.	0.006	n.s.	0.040	0.003

p values for the REML analysis of binding pockets with IgG2 response to FMDV15

	Pocket 4			
Weeks IgG1	13	70	71	74
2	n.s.	<0.001	n.s.	0.021
4	0.016	0.020	n.s.	n.s.
8	0.037	0.004	0.043	n.s.
10	n.s.	n.s.	n.s.	n.s.
AUC	n.s.	0.004	n.s.	n.s.

	position 13				position 70			position 71				position 74		
Weeks IgG1	G	S	K	R	E	R	Q	K	R	A	E	A	E	N
2	n.s.	n.s.	0.031	n.s.	<0.001	<0.001	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.011
4	n.s.	n.s.	0.021	n.s.	0.003	0.006	n.s.	0.024	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
8	n.s.	n.s.	0.016	n.s.	<0.001	<0.001	n.s.	0.004	n.s.	0.044	n.s.	n.s.	n.s.	n.s.
10	n.s.	n.s.	0.016	n.s.	0.024	0.036	n.s.	0.046	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
AUC	n.s.	n.s.	n.s.	n.s.	<0.001	0.001	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.03

p values from the REML analysis of binding pocket 4 and response IgG2 to FMDV15

Appendix C.4

FMDV T Cell Proliferation *DRB3* alleles and pockets

All results from REML model analysis for T cell proliferation response to FMDV15.

T Cell FMDV15	DRB3 alleles																	
WEEK	*0101*	*0201*	*0501*	*0601*	*0701*	*0901*	*0902*	*1001*	*1101*	*1201*	*1501*	*1601*	*1701*	*1801*	*2002*	*2703*	*2707*	*3201*
0	n.s.	n.s.	<0.001	n.s.	n.s.	n.s.	n.s.	n.s.	0.051	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
4	0.014	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
8	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.027	<0.001	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	<0.001	n.s.	n.s.	n.s.
10	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.007	0.004	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

p values from REML analysis of *DRB3* alleles and T cell proliferation in response to FMDV15

Week FMDV15	pocket 1	pocket 6	pocket 7						pocket 9					Pocket 10 pos 56
			28	30	47	61	67	71	9	37	57	60	61	
0	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.006	n.s.	n.s.	n.s.	n.s.	n.s.
4	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.051	n.s.	n.s.	n.s.	n.s.	n.s.
8	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
10	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

: p values from REML analysis of *DRB3* pockets and T cell proliferation in response to FMDV15

Appendix C.5

ConA T Cell Proliferation *DRB3* alleles and pockets

All results from REML model analysis for T cell proliferation response to ConA.

T Cell ConA	DRB3 alleles																	
WEEK	*0101*	*0201*	*0501*	*0601*	*0701*	*0901*	*0902*	*1001*	*1101*	*1201*	*1501*	*1601*	*1701*	*1801*	*2002*	*2703*	*2707*	*3201*
0	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.088	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
4	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.029	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
8	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.004	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
10	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.006	n.s.	n.s.	0.027	n.s.	n.s.	n.s.	n.s.	n.s.

p values from REML analysis of *DRB3* alleles and T cell proliferation in response to ConA

ConA Week	pocket 1	pocket 6	pocket 7						pocket 9					pocket 10
			28	30	47	61	67	71	9	37	57	60	61	
2	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
4	n.s.	n.s.	0.020	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
8	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
10	n.s.	n.s.	0.040	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

p values from REML analysis of *DRB3* pockets and T cell proliferation in response to ConA

Appendix C.6

FMDV15 IFN-γ *DRB3* alleles

All results from GLIMM model analysis for IFN-γ production response to FMDV15 and ConA

IFN-γ FMDV15	DRB3 alleles																	
WEEK	*0101*	*0201*	*0501*	*0601*	*0701*	*0901*	*0902*	*1001*	*1101*	*1201*	*1501*	*1601*	*1701*	*1801*	*2002*	*2703*	*2707*	*3201*
0	n.s.	n.s.	n.s.	n.s.	0.049	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.038	n.s.	n.s.
4	0.013	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	<0.001	0.013	n.s.	0.005	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
8	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.003	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
10	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.034	0.003	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.014	n.s.

p values from GLIMM analysis of *DRB3* alleles IFN-γ levels in response to FMDV15

IFN-γ ConA	DRB3 alleles																	
WEEK	*0101*	*0201*	*0501*	*0601*	*0701*	*0901*	*0902*	*1001*	*1101*	*1201*	*1501*	*1601*	*1701*	*1801*	*2002*	*2703*	*2707*	*3201*
0	n.s.	n.s.	n.s.	n.s.	n.s.	0.053	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.006	n.s.	n.s.
4	<0.001	0.026	<0.001	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.015	n.s.	n.s.	n.s.	n.s.	n.s.	<0.001	n.s.
8	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.027	n.s.	n.s.	0.016	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
10	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

p values from GLIMM analysis of *DRB3* alleles IFN-γ levels in response to ConA

Appendix C.7

Amino acid protein sequence used for 3D modelling

DRA

MAITRVPILGLFITVLIGLQESWAIKENHVIIQAEFYLKPEESAEFMFDFDGDEI

FHVDMGKKETVWRLPEFGHFASFEAQGALANMAVMKANLDIMIKRSNNTP

NTNVPPEVTLLPNKPVELGEPNTLICFIDKFSPPVISVTWLRNGKPVTDGVSQT

VFLPRNDHLFRKFHYLPFLPTTEDVYDCKVEHLGLNEPLLKHWEYEAPAPLP

ETTENAVCALGLIVALVGIIAGTIFIIKGVRKANTVERRGPl

DRB3_1601

MVCLYFSGGSWMAALIVMLMVLCPPLAWAREIQPHFLEYTKKECHFFNGTE

RVRFLDRYFHNGEEFVRFDSDWGEYRAVTELGRPDAKYWNSQKDFLEEKR

AAVDTYCRHNYGVGESFTVQRRVEPIVTVYPAKTQPLQHHNLLVCSVNGFY

PGNIEVRWFRNGHEEEAGVISTGLIQNGDWTFQTMVMLETVPQSGEVYTCQ

VEHPSQTSPITVEWRARSDSAQSKMMSGVGGFVLGLFFLAVGLFIYFRNQKG

RPTLQPTGLLS

Appendix D.1

Probes for DQA Microarray

Oligonucleotide probe	Sequence	base pairs
DQA-A01R2	ATTCTGCGCCGTAGGTG	17
DQA-A04R2	GATGCTTATGCCATAGGTG	19
DQA-A05R3	GAAGTCTGTGCCATAAGT	18
DQA-A06R3	GAAGTCTGCGCCATAAG	17
DQA-A13R2	AGATGTTTATGCCATAGGCA	20
DQA-A14R3	CGTTTATGCCATAGGCA	17
DQA-A16R	ATCACTGTGCCATAGGAG	18
DQA-B09R	TTCCTGGGTGTACTGGTCA	19
DQA-C01R2	AGGTCCACATAAAACATCTC	20
DQA-C02R3	AGATCCACATAAAACATCTC	20
DQA-C03R3	AGGTCCACATAAAACAGCT	19
DQA-C05R3	TCCACATAAAACAACTCGT	19
DQA-C07R3	TTCTAGGTCCACGTAGAA	18
DQA-C08R3	TTCCAGGTCCACGTAGAA	18
DQA-C10R2	TCCACATAAAACAACTCATC	20
DQA-C11R2	TCCACATAAAGCAACTCGT	19
DQA-C12R	GTCCACATAAAACAGCTTGT	20
DQA-D11R	GCCGCCAGACAGTCTCTTT	19
DQA-E01L	GCATGTGTTTAGTAAATTTGCAAGTT	26
DQA-E02L	CCCCTATTTAGCAGAATGTTAAGTT	25
DQA-E06L	TATGTTTAGCCAGTTTGCAGGTTTT	25
DQA-E08L	GCCTATGTTTGATAAATTAAGACGTTTT	28
DQA-E09L	CCTATGTTTGATAAATTACGTTTCCA	26
DQA-E10L2	GCCTATGTTTGATAAATTACGTTTTCA	27
DQA-E11L	CTATGTTTGGTGAATTAACAAGTTTTG	27
DQA-E12L2	CTATGTTTGGTGAATTAACAGGTTTTGA	28
DQA-E13L	TATGTTTGGTGATTTAACAAGTTTTGA	27

DQA-E14L	CTATTTTTAGCCAGTTTGCAGGTT	24
DQA-E15L2	GCCTCTGTTTAGTAAATTTAGACGTT	26
DQA-E16L2	GCCTGTGTTTAGTAAATTTACAAGTTTTG	29
DQA-E17L2	CCTGTGTTTAGTAAATTTGCAACTTTTG	28
DQA-E18L2	CTGTGTTTAGTAAATTTGCAAGTTTT	26
DQA-E20	CCTATGTTTAGCCAGTTTGCAAG	23
DQA-F01L	AGCACAAGATGCGCTGAATGAAA	23
DQA-F02L	AGCACAAGATGCGCTGATGAAA	22
DQA-F08L2	CCGCAAGGTGCACTGAGAAAC	21
DQA-F14	CACAGGCTATACTGAGGAACA	21
DQA-G03S2	AGCAAAACACAACTTGGAT	19
DQA-G04S2	CGAAGCACAATTTGGAGA	18
DQA-G06S2	GTGAAGCACAATTTGGAGA	19
DQA-G07S2	TGAAGCACAATTTGGAGG	18
DQA-G08S2	CATCAAAACACAACTTGGAT	20
DQA-G09S2	CATCAAAACACAATTTGGATAT	22
DQA-G11S2	ACGATGAAGCACAATTTGG	19
DQA-G15S2	TATAGTGAAGCACAATTTGG	20
DQA-G20	TTCATCAAAACACAACTTGGAT	22
DQA-G21	CAGCAAAATTCAACTTGGATC	21
DQA-G22	CTATATCAAAACACAACTTGAATA	24
DQA-H04S2	GATCATGATTCGAAGGTC	18
DQA-H05S2	ATCGTGATTCAAAGGTCC	18
DQA-H07S2	GATCTTGATTCAAAGGTCC	19
DQA-H09S	GGTCATGATTCGAAGGTC	18
DQA-H10S2	GGTCTTGATTCAAAGGTC	18
DQA-H13S2	TCCTGACTAAACGCTACA	18
DQA-H14S	CCTGACTAAACGCTCCAA	18
DQA-H17S	CCTGACTAAACGCTCTAA	18
DQA-H18	GATATCGTGATTCAAAGGTC	20
DQA-H19	GAATATCATGATTAAATACTCCAA	24
DQA Standards		
DQA-S01R	CCAATGTGGTCAGCTGAGTC	20
DQA-S04	CCCTGTTATCAATGGTAAGTGTC	23

Appendix D.2

DQA Microarray probe layout

S3R(1)	B10L2	B14L	B19	B25L2	B30	B36	S2R(1)
B5	B10	B14	B18N	B24L2	B29L	B35	B41
B4L2	B9	B13	B18L	B24L	B28	B34	B42
B4	B8L	B12L	B18	B23	B27	B33L	B40
B3	B8L2	B12	B17L	B22L	B26L2	B32L	B39
B2L	B7	B11N	B16L	B20L	B26L	B31L	B38
B1L2	B5L	B11	B15L	B19L	B25L3	B30L	B37
A3S2	A6N2	A9L	A15	A20	A25	A30	B1
A2S	A6N	A9	A14S2	A19L3	A23L2	A29	A34
A2	A5	A8S2	A13	A19L2	A23	A28	A33
A1S2	A4L2	A8	A12L	A18L2	A22	A27L	A32
A1	A4L	A7L	A11	A17S	A21S2	A27	A31
S2R(2)	A3S	A7	A10	A17	A21	A26S2	S3R(2)

Appendix D.3

DQA allele definition for microarray probes

DQA Allele	DQA-A	DQA-B	DQA-C	DQA-D	DQA-E	DQA-F	DQA-G	DQA-H
*DQA1*0101*	A09R	B07R	C08R3	D03R	E16L2	F11	G05S	H10S2
*DQA1*0102*	A09R	B07R	C08R3	D03R	E16L2	F11	G06S2	H07S2
*DQA1*0103*	A14R3	B07R	C08R3	D03R	E16L2	F11	G10S	H04S2
*DQA1*0201*	A08R2	B07R	C08R3	D02R2	E15L2	F11	G06S2	H05S2
*DQA1*0202*	A09R	B08R2	C08R3	D02R2	E15L2	F11	G06S2	H05S2
*DQA1*0203*	A09R	B07R	C08R3	D02R2	E15L2	F11	G06S2	H05S2
*DQA1*0204*	A13R2	B07R	C08R3	D02R2	E15L2	F11	G04S2	H05S2
*DQA1*0301*	A09R	B07R	C08R3	D02R2	E15L2	F11	G10S	H02
*DQA1*0302*	A09R	B07R	C08R3	D02R2	E15L2	F12	G10S	H03
*DQA1*0401*	A09R	B07R	C08R3	D02R2	E15L2	F11	G05S	H10S2
*DQA1*0801*	A09R	B07R	C06R2	D03R	E18L2	F11	G04S2	H08
*DQA1*0802*	A09R	B07R	C06R2	D03R	E01L	F11	G04S2	H08
*DQA1*10011*	A04R2	B07R	C07R3	D03R	E16L2	F11	G15S2	H05S2
*DQA1*10012*	A04R2	B07R	C08R3	D03R	E16L2	F11	G15S2	H05S2
*DQA1*1002*	A14R3	B07R	C08R3	D03R	E16L2	F11	G15S2	H18
*DQA1*1101*	A09R	B07R	C08R3	D03R	E17L2	F11	G11S2	H05S2
*DQA1*12011*	A04R2	B07R	C08R3	D03R	E17L2	F11	G09S2	H05S2
*DQA1*12012*	A04R2	B07R	C08R3	D03R	E17L2	F11	G10S	H05S2
*DQA1*12021*	A04R2	B07R	C08R3	D03R	E17L2	F11	G11S2	H05S2
*DQA1*12022*	A04R2	B07R	C08R3	D03R	E17L2	F11	G11S2	H05S2
*DQA1*1203*	A04R2	B07R	C08R3	D03R	E17L2	F11	G12	H10S2
*DQA1*1204*	A04R2	B07R	C08R3	D03R	E17L2	F11	G10S	H05S2
*DQA1*1301*	A09R	B07R	C06R2	D01R2	E17L2	F11	G18	H09
*DQA1*1302*	A09R	B07R	C06R2	D01R3	E17L2	F11	G18	H09

*DQA1*1401*	A09R	B07R	C08R3	D03R	E18L2	F11	G19	H09
*DQA2*2001*	A11R	B02R	C01R2	D08R2	E04	F03	G08S2	H14S
*DQA2*2002*	A11R	B02R	C01R2	D08R2	E03S	F04	G08S2	H14S
*DQA2*2101*	A11R	B02R	C01R2	D08R2	E14L	F07L	G08S2	H14S
*DQA2*2201*	A11R	B02R	C01R2	D08R2	E06L	F04	G08S2	H14S
*DQA2*22021*	A11R	B02R	C01R2	D08R2	E06L	F04	G03S2	H14S
*DQA2*22022*	A11R	B02R	C01R2	D06R2	E05L	F04	G03S2	H14S
*DQA2*22023*	A11R	B02R	C01R2	D07R	E05L	F04	G03S2	H14S
*DQA2*22031*	A11R	B02R	C03R3	D07R	E05L	F04	G03S2	H14S
*DQA2*22032*	A11R	B02R	C03R3	D08R2	E06L	F04	G03S2	H14S
*DQA2*2204*	A11R	B02R	C01R2	D08R2	E19	F04	G03S2	H14S
*DQA2*2205*	A10R2	B02R	C01R2	D08R2	E07S	F04	G08S2	H14S
*DQA2*2206*	A11R	B02R	C01R2	D08R2	E06L	F07L	G21	H14S
*DQA2*2401*	A11R	B01R2	C03R3	D08R2	E13L	F05	G09S2	H11S
DQA2 (wsu-2-3)*	A16R	B09R	C12R	D11R	E20	F14	G22	H19
*DQA3*2301*	A12R	B06R	C02R3	D08R2	E12L2	F10	G02S	H12S
*DQA3*25011*	A05R3	B04R2	C04R	D05R2	E09L	F08L2	G14S	H16S
*DQA3*25012*	A05R3	B04R2	C04R	D05R2	E10L2	F08L2	G14S	H15
*DQA3*2502*	A06R3	B04R2	C04R	D05R2	E10L2	F08L2	G13S	H13S2
*DQA3*2601*	A05R3	B04R2	C04R	D05R2	E08L	F09	G17S	H13S2
*DQA3*2602*	A05R3	B04R2	C04R	D05R2	E08L	F08L2	G17S	H13S2
*DQA3*2603*	A05R3	B04R2	C04R	D05R2	E08L	F08L2	G14S	H13S2
*DQA3*27011*	A03R	B06R	C10R2	D09R2	E11L	F01L	G01S	H14S
*DQA3*27012*	A02R2	B05R2	C05R3	D09R2	E11L	F01L	G01S	H14S
*DQA3*2702*	A01R2	B05R2	C10R2	D09R2	E11L	F02L	G01S	H14S
*DQA3*2901*	A15R	B06R	C11R2	D10R	E12L2	F13	G20	H17S
*DQA4*2801*	A07R2	B03R2	C09R	D04R2	E02L	F06	G16S	H01S
New(Y10564)-0102	A09R	B07R	C08R3	D03R	E16L2	F11	G07S2	H10S2

Appendix D.4

Probes for BoLA-Class 1Exon 2 Microarray

Oligonucleotides probe	Sequence	base pairs	Tm ^{o}C
Exon 2, Series A, Codons 62-67			
BoLA-C1Ex2A01	CGGGAGACGCAAAGGGCC	18	57
BoLA-C1Ex2A01S2	CGGGAGACGCAAAGGGC	17	54
BoLA-C1Ex2A02	CAGGAGACGCGAAAGGCC	18	55
BoLA-C1Ex2A02S	GGAGACGCGAAAGGCC	16	51
BoLA-C1Ex2A03S2	CGGAACACGCGAAACGC	17	52
BoLA-C1Ex2A03S	CGGAACACGCGAAACG	16	49
BoLA-C1Ex2A04L	ATCGAAACACGAGAATCTACAA	22	49
BoLA-C1Ex2A04L2	TCGAAACACGAGAATCTACA		
BoLA-C1Ex2A05	GAGCAGACGCGAATAGTC	18	50
BoLA-C1Ex2A06	CGCGAGACGCGAAACTCC	18	55
BoLA-C1Ex2A06N	GACGCGAAACTCCAAGGAA		
BoLA-C1Ex2A07	CGCGAGACTCAAATCTCC	18	50
BoLA-C1Ex2A07L	CGCGAGACTCAAATCTCCA	19	51
BoLA-C1Ex2A8	CGCGAGACGCGAATCTCC	18	55
BoLA-C1Ex2A08S2	CGCGAGACGCGAATCTC	17	52
BoLA-C1Ex2A09	CAGAACACGCGAAACTCC	18	50
BoLA-C1Ex2A10	GAGGAGACGTGGAGAGCC	18	55
BoLA-C1Ex2A11	CAGGAGACGCAGAGAACT	18	50
BoLA-C1Ex2A12L	TCAAGAGACGCGGATACAAA	20	50
BoLA-C1Ex2A13	CAGGCGACGCAGAGAACT	18	53
BoLA-C1Ex2A13S	AGGCGACGCAGAGAACT		
BoLA-C1Ex2A14	CAGGAGACGCGAAACGCC	18	55
BoLA-C1Ex2A14S	GGAGACGCGAAACGCC	16	51
BoLA-C1Ex2A15	GAGATGACACGAGATGCC	18	50
BoLA-C1Ex2A17	GACGAGACGCGAATCTCC	18	53
BoLA-C1Ex2A17S	GACGAGACGCGAATCTC	17	49

BoLA-C1Ex2A18L2	CGCGAAACTTCAAGGACACCG	21	56
BoLA-C1Ex2A19L2	GCGAAACTTAAAGGGCGCCG	20	56
BoLA-C1Ex2A19L3	CGCGAAACTTAAAGGGCGCCG	21	58
BoLA-C1Ex2A20	GCGATGACAAGAGATGCC	18	50
BoLA-C1Ex2A21	CAGAACACGCGAAACGCC	18	53
BoLA-C1Ex2A21S	CAGAACACGCGAAACGC	17	49
BoLA-C1Ex2A22	CAGGAGACGCAGAGGACT	18	53
BoLA-C1Ex2A23	CAGGAGACGCAGATAACT	18	48
BoLA-C1Ex2A23L2	ATCAGGAGACGCAGATAACT	20	50
BoLA-C1Ex2A25	GAGGAAACGCAAAGGGCC	18	53
BoLA-C1Ex2A26S	GAGGAGACGCAAAGGG	16	49
BoLA-C1Ex226S2	GAGGAGACGCAAAGGGC	17	52
BoLA-C1Ex2A27	TCGAAACACGAGGATCTACA	20	50
Exon2, Series B, Codons 72-77			
BoLA-C1Ex2B01	CAGATTTTCCGAGTGAGC	18	48
BoLA-C1Ex2B01L	CAGATTTTCCGAGTGAGCC	19	51
BoLA-C1Ex2B02L2	CAATTTTTCCGAGTGAGCC	19	49
BoLA-C1Ex2B02L	CAATTTTTCCGAGTGAGCCT	20	50
BoLA-C1Ex2B03	CAGACTTTCCGGGCGAAC	18	53
BoLA-C1Ex2B04	CAGAGTTTCCGAGTGAAC	18	48
BoLA-C1Ex2B04L2	CAGAGTTTCCGAGTGAACC	19	51
BoLA-C1Ex2B05	CAGACTTTCCGAGTGGAC	18	50
BoLA-C1Ex2B07	CAGACTTTCCGAGCGAAC	18	50
BoLA-C1Ex2B08	CAGACTTTCCGAGTGTAC	18	48
BoLA-C1Ex2B08L2	ACAGACTTTCCGAGTGTAC	19	49
BoLA-C1Ex2B09	CAGATTTTCCGGGCGAAC	18	50
BoLA-C1Ex2B10	CAGATTTTCCGAGTGGAC	18	48
BoLA-C1Ex2B10L2	ACAGATTTTCCGAGTGGAC	19	49
BoLA-C1Ex2B11	CAGTCTTTCCGAGTGGGC	18	53
BoLA-C1Ex2B11N	ACAGTCTTTCCGAGTGGG		
BoLA-C1Ex2B12	CTGTGGTACCGAGAGGCC	18	55
BoLA-C1Ex2B13	CTGGTGTATCGAGGGAGC	18	53

BoLA-C1Ex2B14	CTGGTGTATCGAGAGAGC	18	50
BoLA-C1Ex2B14L	ACTGGTGTATCGAGAGAGC	19	51
BoLA-C1Ex2B15L	CTGGTATATCGAGAGAGCC	19	51
BoLA-C1Ex2B16	CAATTTTTCCGACGGGGC	18	50
BoLA-C1Ex2B16L	CAATTTTTCCGACGGGGCC	19	53
BoLA-C1Ex2B17L	ACAATTTTTCCGAGTGTACCT	21	49
BoLA-C1Ex2B18	CAGAATTTCCGAGTGGGC	18	50
BoLA-C1Ex2B18N	GAATTTCCGAGTGGGCC		
BoLA-C1Ex2B19	CAGACTTTCCGAGCAAAC	18	48
BoLA-C1Ex2B19L	ACAGACTTTCCGAGCAAAC	19	49
BoLA-C1Ex2B20	AGAGTTTCCGAGTGGGC	17	49
BoLA-C1Ex2B22L	CTGCTGTATCGAGAGAACC	19	51
BoLA-C1Ex2B23	CTGAAGTACCGAGAGGCC	18	53
BoLA-C1Ex2B24L	CAGAAATCCCGATTATGCTTG	21	50
BoLA-C1Ex2B24L2	AGAAATCCCGATTATGCTTGT		
BoLA-C1Ex2B25L2	CAGGAATCCCGATTATGCT	19	49
BoLA-C1Ex2B25L3	AGGAATCCCGATTATGCTTG		
BoLA-C1Ex2B26L	CTGCTGTATCGAAAGAACC	19	49
BoLA-C1Ex2B26L2	CTGCTGTATCGAAAGAACCT	20	50
BoLA-C1Ex2B27	CAGAGATTGCGAACGGGC	18	53
BoLA-C1Ex2B28	CAGACTTTCCGAGTGAAC	18	48
BoLA-C1Ex2B29L	CAGAGATCCCAATTATGCTTG	21	50
BoLA-C1Ex2B30	CAGTCTTTCCGAGTGAAC	18	48
BoLA-C1Ex2B30L	CAGTCTTTCCGAGTGAACC	19	51
BoLA-C1Ex2B31L	CAGTTTCCGAGTGAACCTGA	20	52
BoLA-C1Ex2B31L2	CAGTTTCCGAGTGAACCTG		
BoLA-C1Ex2B32L	CAGGTTTTCCAAGTGAACCT	20	50
BoLA-C1Ex2B33L	CAGGTTTTCCGAGTGAACC	19	51
Class I Exon 2 Standards			
BoLA-C1Ex2S01R	CGTCGCTGTCGAACCG	16	51
BoLA-C1Ex2S02R	GGCGTCGCTGTCGAACCG	18	57

Appendix D.5

Class I exon 2 Microarray probe layout

S4(1)	B16	B20	B25	B30	B35	B42	S3R(1)
B11L	B15	B19L2	B24	B29S2	B34L	B41	C01
B10	B14	B19L	B23S	B29S	B33L2	B40S	B47
B9	B13S	B18L	B23	B28	B33L	B39S	B46
B8	B13	B18	B22S	B27	B32L	B39	B45
B7	B12S	B17L	B21	B26L	B31L	B38L3	B44
B6L	B12	B16S	B20L	B25L	B30L	B38L2	B43
A5R	A9R	A13R	A18R	A22R	A26R	A31R	B6
A4R2	A8R	A12R2	A17R2	A21R	A25R	A30R	B1
A3R	A7R2	A12R	A17R	A20R	A24R	A29R	A34R
A2R	A7R	A11R	A16R	A19R2	A23R2	A28R	A33R
A1R2	A6R2	A10R	A15R	A19R	A23R	A27R2	A32R
S3R(2)	A6R	A9R2	A14R2	A18R2	A22R2	A27R	S4(2)

Appendix D.6

Probes for BoLA-Class 1Exon 3 Microarray

Oligonucleotide Probe	Sequence	base pairs	Tm
Exon 3 Series A Oligos Codons 110-116 (18 bp)			
BoLA-C1Ex3A01R	GGCGTCCTGCCTGTATCC	18	55
BoLA-C1Ex3A02R	GCCGAACTGCTCATAGCC	18	53
BoLA-C1Ex3A03R	GGCGTTCTGCCAGATCCC	18	55
BoLA-C1Ex3A04R	GGCGTCCTGCCTGTACC	17	54
BoLA-C1Ex3A05R	AGCGTCCTGCCTGTACCC	18	55
BoLA-C1Ex3A05R2	AGCGTCCTGCCTGTACC	17	52
BoLA-C1Ex3A06R	GGCGTACTGCCTGTACCC	18	55
BoLA-C1Ex3A07R	GGCGTCCTGACTGTACCC	18	55
BoLA-C1Ex3A07R2	GGCGTCCTGACTGTACC	17	52
BoLA-C1Ex3A08R	GGCGAACTGATCGTACCC	18	53
BoLA-C1Ex3A08R	GGCGAACTGATCGTACCC	17	53
BoLA-C1Ex3A09R	GGCGAGCTGATTATACCCG	19	53
BoLA-C1Ex3A10R	GGCGTCCTGATTATACCCG	19	53
BoLA-C1Ex3A11R	GCCGAACTGCGTATACCC	18	53
BoLA-C1Ex3A12R	GGCGTCCTGCTCATACCC	18	55
BoLA-C1Ex3A13R	GCCGTACTGCTCATACCC	18	53
BoLA-C1Ex3A13R2	GCCGTACTGCTCATACC	17	49
BoLA-C1Ex3A14R	GCCGTACTGATCATACCCG	19	53
BoLA-C1Ex3A14R2	GCCGTACTGATCATACCC	18	50
BoLA-C1Ex3A15R	GGCTAACTGATCATACCCG	19	51
BoLA-C1Ex3A15R2	GGCTAACTGATCATACCC	18	48
BoLA-C1Ex3A16R	GGCGAACTGATCATACCCG	19	53
BoLA-C1Ex3A16R2	GGCGAACTGATCATACCC	18	50
BoLA-C1Ex3A17R	GCCGTACTGCTAATACCCG	19	53
BoLA-C1Ex3A18R	GGCGAACTGCTTGAACCC	18	53
BoLA-C1Ex3A18R2	GGCGAACTGCTTGAACC	17	49
BoLA-C1Ex3A19R	GCCGAACTGCGTGAACCC	18	55

BoLA-C1Ex3A19R2	GCCGAACTGCGTGAACC	17	52
BoLA-C1Ex3A20R	GGCGTCCTGCATGAACCC	18	55
BoLA-C1Ex3A20R2	GGCGTCCTGCATGAACC	17	52
BoLA-C1Ex3A21R	GCCGTACTGCATGAACCC	18	53
BoLA-C1Ex3A21R2	GCCGTACTGCATGAACC	17	49
BoLA-C1Ex3A22R	GGCGAACTGCATGAACCC	18	53
BoLA-C1Ex3A22R2	GGCGAACTGCATGAACC	17	49
BoLA-C1Ex3A23R	GCCGAACTGCATGAACCC	18	53
BoLA-C1Ex3A23R2	GCCGAACTGCATGAACC	17	49
BoLA-C1Ex3A24R	GCCGAACTGCCAGAACCC	18	55
BoLA-C1Ex3A25R	GCCGAACTGCCAAAACCC	18	53
BoLA-C1Ex3A26R	GGCGAACTGATCATACCGC	19	53
BoLA-C1Ex3A27R	GGCCTTCTGCCAGAATCCA	19	53
Exon 3 Series B Oligos Codons 151-157			
BoLA-C1Ex3B01	CGCTGAGGAGAGACACAC	18	53
BoLA-C1Ex3B06	GCAGGCAAAGATCCAACG	18	50
BoLA-C1Ex3B06L	GGCAGGCAAAGATCCAACG	19	53
BoLA-C1Ex3B07	GGAGGCAGAGTTCCAACG	18	53
BoLA-C1Ex3B08	TAATGCGGAGAGCGAGAG	18	50
BoLA-C1Ex3B09	TAATGCGGAGAGCGGGAG	18	53
BoLA-C1Ex3B09S	TAATGCGGAGAGCGGGA	17	49
BoLA-C1Ex3B10	TCGTGCGGAGAGATTCAG	18	50
BoLA-C1Ex3B11L	GTGAAGCTGAGGTACAGAG	19	51
BoLA-C1Ex3B12	TGAGGCGGAGAGACAGAG	18	53
BoLA-C1Ex3B12N	TGAGGCGGAGAGACACAG	18	53
BoLA-C1Ex3B13	TGAGGCGGAGAGACGCAG	18	55
BoLA-C1Ex3B13S	TGAGGCGGAGAGACGCA	17	52
BoLA-C1Ex3B15	TGAGGCGGAGAGATTCAG	18	50
BoLA-C1Ex3B16	TGATGCCGCGCGTGTGAG	18	55
BoLA-C1Ex3B16S	TGATGCCGCGCGTGTGA	17	52
BoLA-C1Ex3B17	TGATGCGGAGACTTGGAG	18	50
BoLA-C1Ex3B17L	GTGATGCGGAGACTTGGAG	19	53
BoLA-C1Ex3B18	TGATGCGGAGAGACAGAG	18	50
BoLA-C1Ex3B18L	GTGATGCGGAGAGACAGAG	19	53

BoLA-C1Ex3B19	TGATGCGGAGAGATTAAG	18	46
BoLA-C1Ex3B19L	GGTGATGCGGAGAGATTAAG	20	52
BoLA-C1Ex3B19L2	GTGATGCGGAGAGATTAAG	19	49
BoLA-C1Ex3B20	TGATGCGGAGAGATTCAG	18	48
BoLA-C1Ex3B20L	GTGATGCGGAGAGATTCAG	19	51
BoLA-C1Ex3B21	TGATGCGGAGGGACACAG	18	53
BoLA-C1Ex3B22	TGATGCGGCGCGTGTGAG	18	55
BoLA-C1Ex3B22S	TGATGCGGCGCGTGTGA	17	52
BoLA-C1Ex3B23	TGCTGCGAAGGGCGAGAG	18	55
BoLA-C1Ex3B23S	TGCTGCGAAGGGCGAGA	17	52
BoLA-C1Ex3B24	TGCTGCGGAGACTTGGAG	18	53
BoLA-C1Ex3B25	TGCTGCGGAGAGACAGAG	18	53
BoLA-C1Ex3B25L	TGCTGCGGAGAGACAGA	17	49
BoLA-C1Ex3B26	TGCTGCGGAGAGATTAAG	18	48
BoLA-C1Ex3B26L	GTGCTGCGGAGAGATTAAG	19	51
BoLA-C1Ex3B27	TGCTGCGGAGAGATTCAG	18	50
BoLA-C1Ex3B28	TGCTGCGGAGCGTGTGAG	18	55
BoLA-C1Ex3B29S	TGCTGCGGAGGGCGAGA	17	54
BoLA-C1Ex3B30	TGTTGCGGAGAGATTCAG	18	48
BoLA-C1Ex3B30L	GTGTTGCGGAGAGATTCAG	19	51
BoLA-C1Ex3B31L	GTTACGCTGAGGTACAGAG	19	51
BoLA-C1Ex3B32	TTATGCTGAGGTACAGAG	18	46
BoLA-C1Ex3B32L	GTTATGCTGAGGTACAGAG	19	49
BoLA-C1Ex3B33L	CAGATTATGCTGAGTCTTTGA	21	49
BoLA-C1Ex3B33L2	CAGATTATGCTGAGTCTTTG	20	48
BoLA-C1Ex3B34L	GGTTCTACGGACTTTTACAG	20	50
BoLA-C1Ex3B35	TTCTGCGGAGAGCGGGAG	18	55
BoLA-C1Ex3B35S	TTCTGCGGAGAGCGGGA	17	52
BoLA-C1Ex3B38L	AAGGTTATGCTGAGTCTTTGA	21	49
BoLA-C1Ex3B38L2	AAGGTTATGCTGAGTCTTTG	20	48
Class 1 Exon 3 Standards			
BoLA-C1Ex3S01R	GTCCTCGTTCAGGGCGA	17	52
BoLA-C1Ex3S2	TCGCCCTGAACGAGGACCTG	20	58
BoLA-C1Ex3S2R	CAGGTCCTCGTTCAGGCGA	20	58

Appendix D.7

Class I exon 3 Microarray probe layout

S041	G13S	F11	E15L2	D10R	C06R2	A15R	S01R1
H08	G14S	F12S	E16L2	D11R	C07R3	A16R	A01R2
H09	G15S2	F13	E17L2	E01L	C08R3	B01R2	A02R2
H09S	G16S	F14	E18L2	E02L	C09R	B02R	A03R
H10S2	G17S	G01S	E19	E03S	C10R2	B03R2	A04R2
H11S	G18	G02S	E20	E04	C11R2	B04R2	A05R3
H12S	G19	G03S2	F01L	E05L	C12R	B05R2	A06R3
H13S2	G20	G04S2	F02L	E06L	D01R2	B06R	A07R2
H14	G21	G05S*	F03	E07S	D02R2	B07R	A08R2
H14S	G22	G06S2	F04	E08L	D03R	B08R2	A09R
H15	H01S	G07S2	F05	E09L	D04R2	B09R	A10R2
H16S	H02	G08S2	F06	E10L2	D05R2	C01R2	A11R
H17S	H03	G09S2	F07L	E11L	D06R2	C02R3	A12R
H18	H04S2	G10S	F08L2	E12L2	D07R	C03R3	A13R2
H19	H05S2	G11S2	F09	E13L	D08R2	C04R	A14R3
S01R2	H07S2	G12	F10	E14L	D09R2	C05R3	S042

Appendix D. 8

MHC Class I probe definitions for each haplotype

AH-02	AH-03	AH-07	AH-10	AH-11	AH-12	AH-13	AH-14	AH-15	AH-06	AH-18
Exon 2 A probes										
ExA03S	Ex2A19L2	Ex2A02	ExA02	Ex2A01	Ex2A06N	Ex2A02	Ex2A04L	Ex2A02	Ex2A02	Ex2A02
ExA03S2	Ex2A19L3	Ex2A02S	Ex2A02S	Ex2A01S	Ex2A06N2	Ex2A02S	Ex2A04L2	Ex2A02S	Ex2A02S	Ex2A02S
ExA04L	Ex2A21	Ex2A21	Ex2A03S	Ex2A03S	Ex2A08	Ex2A04L	Ex2A05	Ex2A04L	Ex2A07	Ex2A07
ExA04L2	Ex2A21S2	Ex2A21S2	Ex2A03S2	Ex2A03S2	Ex208S2	Ex2A04L2	Ex2A08	Ex2A04L2	Ex2A07L	Ex2A07L
ExA08		Ex2A22	Ex2A09	Ex2A04L		Ex2A05	Ex2A08S2	Ex2A05		Ex2A11
ExA08S2			Ex2A09L	Ex2A04L2		Ex2A08	Ex2A13	Ex2A08		
			Ex2A18			Ex2A08S2	Ex2A25	Ex2A08S2		
			Ex2A21			Ex2A13		Ex2A13		
			Ex2A21S			Ex2A25		Ex2A27		
			Ex2A22					Ex2A27L		
Exon 2 B probes										
Ex2B04	Ex2B05	Ex2B04	Ex2B02L	Ex2B01L2	Ex2B13	Ex2B05	Ex2B08L	Ex2B08L	Ex2B12	Ex2B04
Ex2B04L2	Ex2B05L	Ex2B04L	Ex2B05	Ex2B09		Ex2B05L	Ex2B08L2	Ex2B08L2	Ex2B12L	Ex2B04L2

Ex2B05	Ex2B07	Ex2B05	Ex2B05L	Ex2B31L		Ex2B18	Ex2B14	Ex2B13	Ex2B23	Ex2B17L
Ex2B05L		Ex2B05L	Ex2B31			Ex2B18N	Ex2B14L			Ex2B23
Ex2B08L		Ex2B31L				Ex2B18L	Ex2B24L			Ex2B24L
Ex2B08L2						Ex2B25L2	Ex2B24L2			Ex2B24L2
						Ex2B25L3	Ex2B33L			
						Ex2B26L				
						Ex2B26L2				

AH-02	**AH-03**	**AH-07**	**AH-10**	**AH-11**	**AH-12**	**AH-13**	**AH-14**	**AH-15**	**AH-06**	**AH-18**
Exon 3 **A probes**										
Ex3A13R	Ex3A04R2	Ex3A09R	Ex3A01R2	Ex3A13R	Ex3A14R2	Ex3A04R2	Ex3A07R	Ex3A04R2	Ex3A11R	Ex3A13R
Ex3A18R	Ex3A21R	Ex3A09R2	Ex3A04R2	Ex3A21R	Ex3A20R	Ex3A13R	Ex3A07R2	Ex3A07R		Ex3A16R
Ex3A18R2		Ex3A21R	Ex3A05R	Ex3A22R	Ex3A	Ex3A22R	Ex3A09R	Ex3A07R2		
Ex3A21R			Ex3A09R	Ex3A22R2		Ex3A27R	Ex3A09R2	Ex3A09R		
Ex3A23R			Ex3A09R2			Ex3A27R2	Ex3A25R	Ex3A09R2		
Ex3A23R2			Ex3A17R					Ex3A25R		
			Ex3A21R							
			Ex3A23R							
			Ex3A23R2							

Exon 3 B probes										
Ex3B08	Ex3B01	Ex3B19L	Ex3B11L	Ex3B12	Ex3B06	Ex3B06	Ex3B09	Ex3B16	Ex3B18	Ex3B12
Ex3B24	Ex3B33L	Ex3B19L2	Ex3B19L	Ex3B12S	Ex3B06L	Ex3B06L	Ex3B1623S	Ex3B16S	Ex3B18L	Ex3B12S
Ex3B32L	Ex3B33L2	Ex3B29S	Ex3B19L2	Ex3B23	Ex3B17L	Ex3B33L	Ex3B23S	Ex3B23		Ex3B16
		Ex3B29S2	Ex3B29S	Ex3B23S	Ex3B30	Ex3B33L2	Ex3B29S	Ex3B 23S		Ex3B 16S
		Ex3B32L	Ex3B29S2	Ex3B25	Ex3B30L		Ex3B29S2	Ex3B 29S		Ex3B 19L
			Ex3B32	Ex3B25L			Ex3B38L2	Ex3B38L2		Ex3B19L2
			Ex3B39	Ex3B31L			Ex3B38L3	Ex3B 38L3		Ex3B 27
			Ex3B39S							

AH-19	AH-20	AH-31	AH-33	AH-44	AH-60	AH-61	AH-62	AH-63	AH-64	AH-65
Exon 2 A probes										
Ex2A02	Ex2A02	Ex2A05	Ex2A03S	Ex2A04	Ex2A06N	Ex2A02	Ex2A03S	Ex2A02	Ex2A10	Ex2A08
Ex2A02S	Ex2A02S	Ex2A08	Ex2A03S2	Ex2A04L2	Ex2A06N2	Ex2A02S	Ex2A03S2	Ex2A02S	Ex2A28	Ex2A08S2
Ex2A04L	Ex2A03S	Ex2A08S2	Ex2A04L	Ex2A21	Ex2A08	Ex2A07	Ex2A04L04L2	Ex2A05		Ex2A11
Ex2A04L2	Ex2A03S2	Ex2A13	Ex2A04L2	Ex2A21S	Ex2A08S	Ex2A07L	Ex2A07	Ex2A07		Ex2A28
Ex2A07	Ex2A08		Ex2A13			Ex2A11	Ex2A07L	Ex2A07L		
Ex2A07L	Ex2A08S2					Ex2A12L	Ex2A11	Ex2A08		
	Ex2A22							Ex2A08S2		
								Ex2A19L2		
								Ex2A19L3		
								Ex2A22		
								Ex2A28		
Exon 2 B probes										
Ex2B08L	Ex2B05	Ex2B11	Ex2B04	Ex2B18	Ex2B13	Ex2B12	Ex2B04	Ex2B03		Ex2B17L
Ex2B08L2	Ex2B05L	Ex2B11N	Ex2B04L2	Ex2B18N		Ex2B12L	Ex2B04L2	Ex2B05		Ex2B35
Ex2B23	Ex2B20L	Ex2B13	Ex2B10	Ex2B18L			Ex2B23	Ex2B05L		Ex2B36
Ex2B25L2	Ex2B31L	Ex2B22L	Ex2B10L2	Ex2B24L			Ex2B34	Ex2B13		

Ex2B25L3		Ex2B24L	Ex2B24L	Ex2B24L2				Ex2B17L		
		Ex2B24L2	Ex2B24L2					Ex2B23		
								Ex2B28		
								Ex2B30		
								Ex2B30L		
								Ex2B36		

AH-19	AH-20	AH-31	AH-33	AH-44	AH-60	AH-61	AH-62	AH-63	AH-64	AH-65
Exon 3 A probes										
Ex3A13R	Ex3A09R	Ex3A06R	Ex3A21R	Ex3A17R	Ex3A09R	Ex3A13R	Ex3A09R	Ex3A04R2	Ex3A02R	Ex3A06R
Ex3A17R	Ex3A09R2	Ex3A06R2	Ex3A24R	Ex3A17R2	Ex3A09R2		Ex3A09R2	Ex3A07R	Ex3A05S	Ex3A06R2
Ex3A17R2	Ex3A13R	Ex3A12R		Ex3A20R	Ex3A19R2		Ex3A21R	Ex3A07R2		Ex3A09R
Ex3A19R	Ex3A21R	Ex3A12R2		Ex3A22R	Ex3A20R		Ex3A29R	Ex3A13R		Ex3A09R2
Ex3A19R2				Ex3A22R2			Ex3A31R	Ex3A14R2		
Ex3A 23R								Ex3A29R		
Ex3A23R2								Ex3A30R		
Ex3A27R								Ex3A32R		
Ex3A27R2										
Exon 3 B probes										
Ex3B19L	Ex3B19L	Ex3B20	Ex3B24	Ex3B19L	Ex3B14	Ex3B19L	Ex3B27	Ex3B19L	Ex3B34L	Ex3B16
Ex3B19L2	Ex3B19L2	Ex3B20L	Ex3B32L	Ex3B19L2	Ex3B17L	Ex3B19L2	Ex3B32L	Ex3B19L2		Ex3B16S
Ex3B33L	Ex3B33L	Ex3B23		Ex3B21		Ex3B27	Ex3B38L2	Ex3B27		Ex3B30
Ex3B33L2	Ex3B33L2	Ex3B23S		Ex3B33L			Ex3B38L3	Ex3B29S		Ex3B30L
		Ex3B26L		Ex3B33L2				Ex3B29S2		
								Ex3B30		

								Ex3B30L		
								Ex3B40S		

AH-66	AH67	AH68	MISC.
Exon 2 A probes			
Ex2A02	Ex2A02	Ex2A02	Ex2A14S2
Ex2A02S	Ex2A02S	Ex2A02S	Ex2A23
Ex2A03S	Ex2A04L	Ex2A03S	Ex2A23L2
Ex2A03S2	Ex2A04L2	Ex2A03S2	Ex2A26S2
	Ex2A11	Ex2A05	Ex2A28
	Ex2A25	Ex2A07	
		Ex2A07L	
Exon 2 B probes			
Ex2B03	Ex2B05	Ex2B04	Ex2B13L
Ex2B04	Ex2B08L	Ex2B04L2	Ex2B16L
Ex2B04L2	Ex2B08L2	Ex2B05	Ex2B28
Ex2B05	Ex2B17L	Ex2B05L	Ex2B29
Ex2B05L	Ex2B33L	Ex2B23	Ex2B32

AH-66	AH67	AH68	MISC.
Exon 3 A probes			
Ex3A19R	Ex3A13R	Ex3A07R	Ex3A03R
Ex3A19R2	Ex3A25R	Ex3A07R2	Ex3A08R
Ex3A21R		Ex3A13R	Ex3A10R
Ex3A29R		Ex3A19R	
Ex3A30R		Ex3A19R2	
Ex3A32R		Ex3A21R	
		Ex3A29R	
Exon 3 B probes			
Ex3B19L	Ex3B09	Ex3B27	Ex3B07
Ex3B19L2	Ex3B27	Ex3B32L	Ex3B10
Ex3B32L		Ex3B33L	Ex3B13
Ex3B40S		Ex3B33L2	Ex3B13S
		Ex3B38L2	Ex3B28
		Ex3B38L3	
		Ex3B40S	

Appendix D.9

DQA potential duplication based on *DRB3* alleles

DRB3 Allele	Potential DQ duplication	Reference
**0101*	N	(Glass, Oliver, & Russell 2000)
**0201*	N	(Park et al. 2004)
**0501*	N	(Sigurdardottir et al. 1992)
**0601*	Y	(Park et al. 2004)
**0701*	Y	(Sigurdardottir et al. 1992)
**0901*	Y	(Sigurdardottir et al. 1992)
**0902*	N	(Park et al. 2004)
**1001*	Y	(Park et al. 2004)
**1101*	Y	(Park et al. 2004)
**1201*	Y	(Glass, Oliver, & Russell 2000; Park J.Y. et al. 2004)
**1202*	Y	(Sigurdardottir et al. 1992)
**1501*	Y	(Park et al. 2004)
**1601*	Y	(Sigurdardottir et al. 1992)
**1701*	Y	(Park et al. 2004)
**2002*	N	(Glass, Oliver, & Russell 2000)
**2703*	Y	(Park et al. 2004)
**3201*	Y	(Sigurdardottir et al. 1992)